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13. ABSTRACT (Maximum 200 Words)

The goal of the work proposed in this application, which has just completed Year 1, was to analyze the ability of the p53 tumor suppressor protein to repress the anti-apoptotic genes survivin and cIAP-2. In particular, we proposed to clone and characterize the cIAP-2 promoter, and to use DNA footprinting on the survivin promoter to look for p53 association in vivo. This has been accomplished. Additionally, we proposed to determine the mechanism whereby the survivin gene is over-expressed in breast tumor cells relative to non-transformed breast cells (MCF-10F). We have determined that the survivin promoter is E2F-responsive; this transcription factor is de-regulated in many breast carcinomas, as well as other tumor types. We show that the E2F site in the survivin promoter is responsible for the enhanced expression of survivin in breast carcinomas versus non-transformed breast epithelial cells. All of the goals for Year 1 have been accomplished; part of this work has been published in the manuscript presented in the Appendix (Bao et al., 2002); the majority is in preparation for submission. We are moving forward to Task 2 (years 12-36), the generation of a replication-selective adenovirus that has the E1A gene driven by the survivin promoter.

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Progress Report
ARMY grant DAMD-17-02-1-0383
Maureen E. Murphy, Ph.D.

I. Introduction

The goal of the work proposed in this application, which has just completed Year 1, is to analyze the ability of the p53 tumor suppressor protein to transcriptionally repress the anti-apoptotic genes *survivin* and *cIAP-2*. In particular, we sought to identify the mechanism whereby p53 represses these genes, using techniques such as deletion mapping of promoter constructs, reporter assays, and DNA footprinting. We have found three key results: first, that p53 directly represses the *survivin* gene by binding to a novel DNA binding site (a variation of the canonical p53-binding site), and displacing the E2F transcription factor, which binds to an overlapping binding site. Second, that p53 also transcriptionally represses cIAP-2; we have cloned the cIAP-2 promoter, and show that it contains two p53 binding sites that are similar to that in the *survivin* promoter. Third, that survivin is over-expressed in breast carcinomas, but not non-transformed breast epithelial cells, by virtue of the fact that this gene is E2F-responsive. E2F transcription factors are de-regulated in many tumor types, including breast carcinomas. This information provides the critical background we needed to complete Task 2 of this work; the generation of an adenovirus that contains the E1A gene (essential for viral replication) driven by the p53-repressible *survivin* promoter. Such a virus would be predicted to selectively replicate in breast carcinomas that have inactivating mutations in p53. We are poised to test this prediction.

II. Body

A. Overview of Progress, Year 1: We have either completely accomplished, or made great strides and are close to accomplishing, every Aim described in Task 1, which covered year 1 (months 1-12). In terms of publications, the manuscript that comprised the bulk of Preliminary Data for this proposal (which was submitted at the time the proposal was written) has been accepted, and a copy is included in the Appendix (Hoffman et al., 2002). Additionally, part of the work described in Task 1 below has been submitted and published in J. Natl Cancer Institute (Bao et al., 2002); this manuscript is also included in the Appendix. The majority of the data we generated in Year 1 is in preparation for submission; we expect it to be submitted by the end of the summer of 2003 (August 2003).

In our proposal we proposed to perform in vivo footprinting of the survivin promoter, in order to define those sites bound by transcription factors in the presence of p53. Using a combination of footprinting as well as chromatin immunoprecipitation, **we have succeeded in this effort, and showed that p53 binds to an element we call the NRE** (negative response element) in vivo. This NRE consists of a modified canonical p53 binding site, with a three-base spacer in between the two ten-base pair binding sites (see enclosed reprint, Hoffman et al., 2002).

We also proposed to define the element necessary for survivin overexpression in human tumors; we called this the tumor reactivation element (TRE). Using site-directed mutagenesis, **we defined the TRE in the survivin promoter to be an E2F-binding site**; this binding site overlaps with the NRE (negative response element), so our conclusion is that the NRE and TRE sites in the *survivin* promoter are distinct but overlapping.

Finally, **we cloned the promoter for the other IAP (inhibitor of apoptosis) that we found was repressed by p53, cIAP-2.** Sequence analysis of the cIAP-2 promoter and intronic regions revealed two p53-binding sites. We have mutated these p53-binding sites of the cIAP-2 promoter, and are testing the requirement for these for repression by p53. Plans are still underway for in vivo footprinting of the cIAP-2 promoter.

B. Detailed Progress:

Task 1. **To define the minimal element in the survivin promoter necessary and sufficient for negative regulation by p53 (the p53-negative response element, or p53 NRE). To define the minimal tumor-reactivation element (TRE), necessary for survivin re-expression in human tumors. To clone and characterize the cIAP-2 promoter.**

- In vivo footprinting of the survivin promoter; definition of the p53 binding site (the NRE, months 1-12).

We performed in vivo footprinting of the *survivin* promoter in cells with inducible p53. As can be seen in Figure 1A (left four lanes), strong secondary structure of this promoter initially hampered our efforts. However, we had significant success when we chose to perform in vitro footprinting of this promoter, using purified p53 (Figure 1B). We complemented these studies with chromatin immunoprecipitation (ChIP) analysis, which measures transcription factor association in vivo (see below).

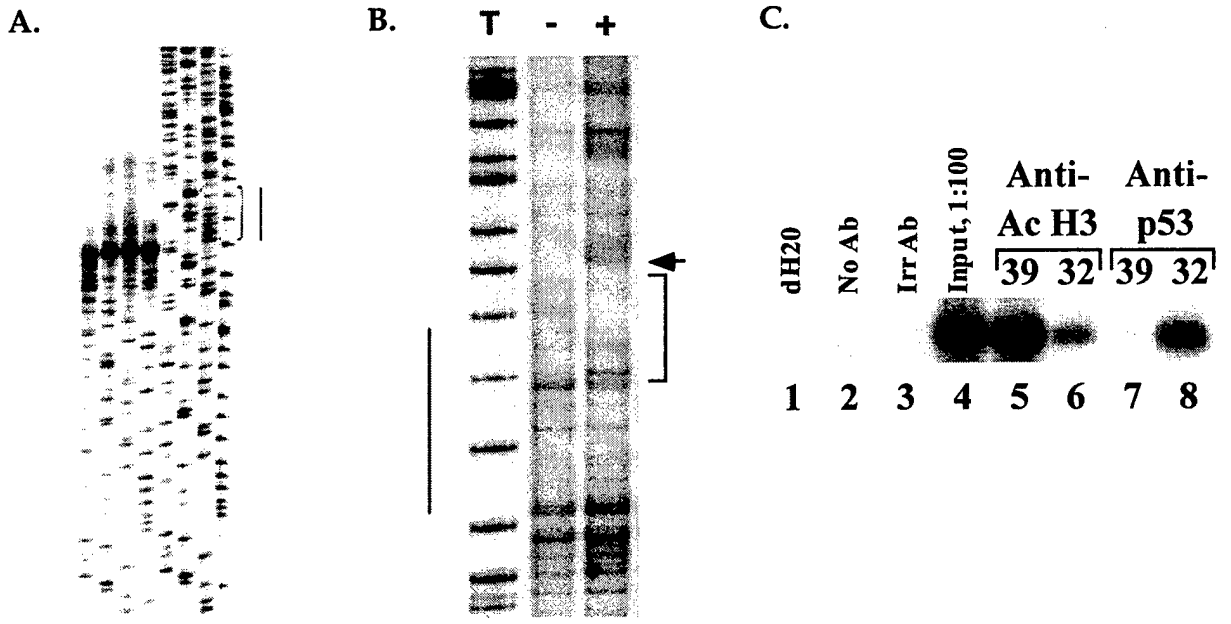

Figure 1. Footprinting and Chromatin Immunoprecipitation (ChIP) analysis of the survivin promoter bound to wild type p53.
A. Initial attempts to footprint the survivin promoter revealed extensive secondary structure in this region, near the p53 binding site. The p53 binding site is denoted by the side-bar; the intense bands in the left four lanes are indiciative of strong secondary structure.
B. In vitro footprinting of the survivin promoter with p53 reveals a region protected by DNAse I digestion in the presence of wild type p53. The p53 binding site is denoted by the left side-bar; the footprinted (protected) region is denoted by the bracket. A hyper-sensitive site is denoted by the arrow. T: sequencing reaction, - : minus p53, +: plus p53.
C. Chromatin IPs using antisera for p53, and primers specific for the survivin promoter, indicate that only wild type p53 (32 degrees) and not mutant p53 (39 degrees) associates with the survivin promoter in vivo (lane 8). Association of p53 with the survivin promoter at 32 degrees correlates with decreased histone H3 acetylation at this promoter, which is consistent with histone deacetylase (HDAC) activity (compare lanes 5 and 6).

As depicted in Figure 1B, we show that p53 binds to the *survivin* promoter to a site that overlaps the binding site we identified initially. This identification of the p53 binding site was supported by chromatin immunoprecipitations, which demonstrate that p53 binds to the *survivin* promoter in vivo (Figure 1C).

- Define potential promoter elements influential in the re-expression of this gene in tumors (the tumor reactivation element, TRE). (months 1-8).

Using deletion analyses of the *survivin* promoter, we showed that the tumor reactivation element of the survivin promoter (TRE) **overlaps with an E2F binding site in this promoter**. To show this, we cloned the *survivin* promoter linked to the assayable reporter gene for firefly luciferase (SP-LUC) and compared *survivin* promoter activity in MCF10F cells (non-transformed mammary epithelial cells, obtained courtesy of Jose Russo, M.D., Fox Chase Cancer Center) versus several human and mouse breast carcinoma cell lines. These data are depicted in Figure 2; they indicate that this promoter is barely functional in non-transformed MCF10F cells, but it maintains robust activity in human breast carcinoma cell lines (see Figure 2). Notably, a minimal survivin promoter construct, containing only the E2F and overlapping p53 binding sites (SP min-LUC), shows the same results (Figure 2, right lanes). We found similar results in other tumor

types, including ovarian cancers. Specifically, non-transformed ovarian cells (HOSE, human ovarian surface epithelium) barely drive expression from the *survivin* promoter, but ovarian tumor cell lines maintain robust expression from this promoter (see manuscript in the Appendix; Bao et al., J Natl Cancer Institute 94: 522-528, 2002). Our analysis of the *survivin* promoter (footprinting, and over-expression in breast carcinomas) is in preparation for submission.

- Cloning of the cIAP-2 promoter (months 1-12).

We have cloned the cIAP-2 promoter, and have found that the fragment we cloned of approximately 3 kilobases is sufficient to confer negative regulation by p53 in stably-transfected cells with inducible p53 (see Figure 3). Sequence analysis of this region includes two canonical p53 binding sites (one in the promoter, and one in the first intron, with zero and two mismatches from the canonical p53-binding site, respectively). We are currently mutating each of these sites individually (focusing on mutated the conserved, essential C and G residues in the site), as well as in combination, to test the requirement for these sites for negative regulation by p53. Interestingly, we have found that p53 is able to repress cIAP-2 even in the presence of inhibitors of new protein synthesis (cycloheximide, data not shown), indicating that p53 directly negatively regulates cIAP-2.

- Deletion analysis and site-directed mutagenesis of the *survivin* promoter, to further define the minimal p53-negative-regulatory element (p53 NRE) and tumor reactivation element (TRE) (months 8-12).

We have defined the minimal *survivin* promoter that is capable of repression by p53. Our data indicate that the p53 NRE, and the TRE (tumor reactivation element, which is an E2F binding site) are both required for repression of the *survivin* promoter by p53; therefore, our current model is that p53 negatively regulates *survivin* by displacing E2F. This fact has significance for Task 2 (generation of a replication-deficient adenovirus controlled by the survivin promoter). Specifically, our data indicate that the minimal *survivin* promoter is both E2F-responsive as well as p53-repressible. Therefore, a genetically engineered adenovirus with the E1A gene (essential for viral replication) driven by the minimal *survivin* promoter would be predicted to have enhanced cytotoxicity not only to tumors with inactive p53, but also to tumors with inactivating mutations in the RB pathway. **Such a virus would be predicted to have even greater selective toxicity to tumor cells relative to normal cells.** We have recently cloned the viral E1A gene upstream of the minimal *survivin* promoter (in single copy). We are testing the p53-repression of this construct, as well as E2F inducibility, and we plan to use this construct for construction of our novel, selectively-cytotoxic adenovirus, as described in Task 2.

Goals for the next year: We plan to create our recombinant adenovirus, as described in Task 2. Additionally, because we have made such rapid progress on Task 1, we plan to extend Task 2 to include other gene therapeutic protocols. Specifically, we have recently found that the arginine 72 polymorphism of p53 has greatly enhanced ability to induce apoptosis than the form of p53 most commonly studied, the proline 72 form (Dumont et al., Nature Genetics 2003). **We plan to create adenoviruses that express either the proline 72 form, or the arginine 72 form, and compare the ability of these two proteins to induce apoptosis in infected cells. This added Aim will not replace any in the original proposal; it merely extends upon the work we have already done, and are planning on doing.** Further, it extends upon the gene therapy theme of our work.

Figure 2.

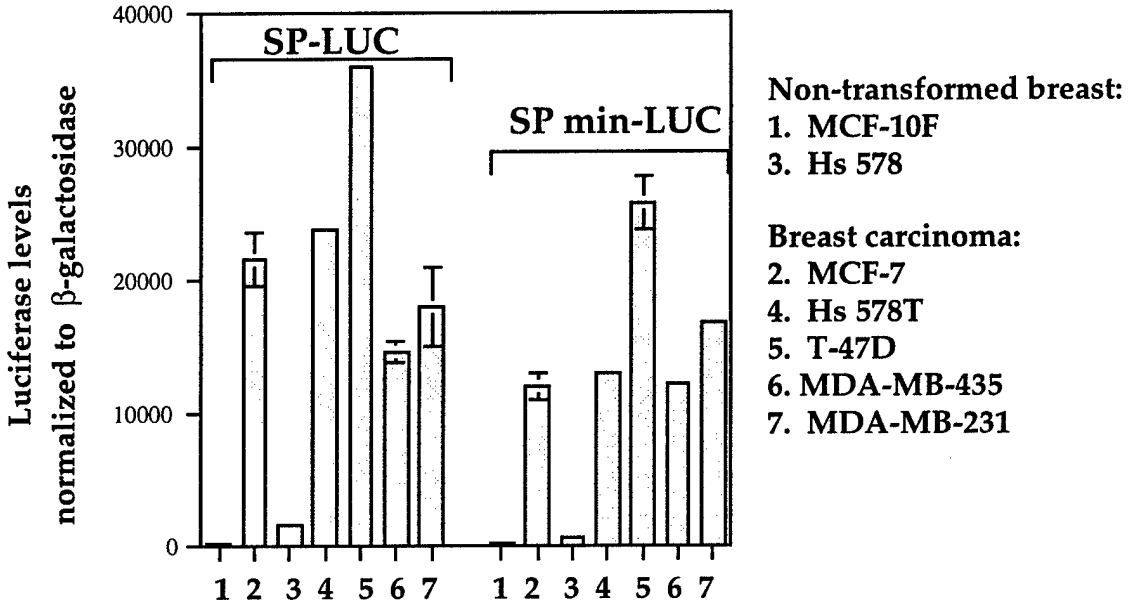

Figure 2. Level of activity of the transfected luciferase gene, driven by the full length survivin promoter, or the minimal p53-repressible promoter (SP min-LUC) in normal (lanes 1 and 3) and transformed (lanes 2, 4-7) breast carcinoma cell lines. Data depicted are the averages of three independent experiments, normalized to a co-transfected β-galactosidase control.

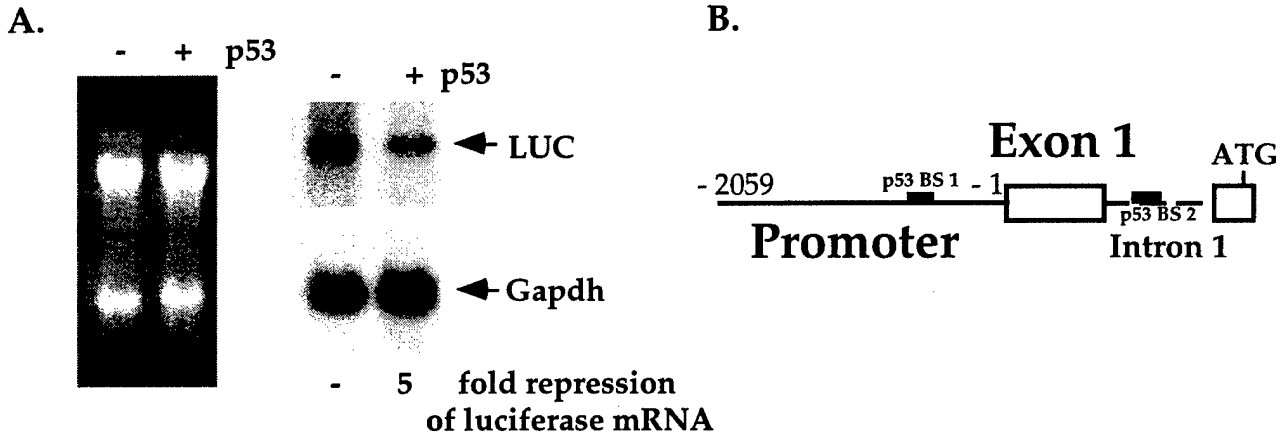

Figure 3. The human cIAP-2 promoter is negatively regulated by p53.
A. Northern analysis of the stably-transfected luciferase gene, driven by the cIAP-2 promoter, in cells with inducible p53. In the lane marked "+", p53 has been induced for 24 hours.
B. Genomic structure of the cIAP-2 promoter analyzed in A; canonical p53-binding sites are denoted.

III. Key Accomplishments:

- Identification of the minimal p53-responsive *survivin* promoter.

- Footprinting and chromatin immunoprecipitation analyses of the *survivin* promoter, demonstrating that p53 binds to the NRE (negative response element).

- Generation of assayable reporter genes, luciferase and SEAP, driven by the survivin promoter. Because SEAP is secreted, cell media, or blood samples from transgenic mice, can be assayed for this product.

- Identification of the TRE of the survivin promoter (the element responsible for high expression of this gene in tumor, and not normal, cells) as an E2F-response element that is nested within the p53 binding site.

- Cloning of the cIAP-2 promoter. Identification of two p53 binding sites within this promoter.

-Construction of cell lines with inducible p53, stably transfected with a cIAP-2 promoter-luciferase construct.

IV. Reportable Outcomes

Manuscripts: Bao et al., J Natl Cancer Institute 94: 522-528, 2002.

Cell lines: H1299 cIAP2-Luc. Has the Luciferase reporter gene driven by the cIAP-2 promoter, stably integrated into cells with inducible p53.

Plasmid constructs: pSRVN-SEAP. Has the secreted for of alkaline phosphatase driven by the survivin promoter. A useful reporter for analysis of tumor cell lines. Has been requested by over 10 Investigators since publication in Bao et al.

V. Conclusions

We have completed our analysis of the *survivin* promoter, and are quite close to finishing our analysis of the cIAP-2 promoter. We are currently determining if p53 represses both genes via the same mechanism; that is, binding to canonical sites and recruiting the p53-Sin3-HDAC complex, which deacetylates histones associated with promoter regions. Notably, we have defined the minimal p53-repressible element in the survivin promoter, and are poised to use this element to design a selectively replicating adenovirus that has a p53-repressible E1A gene. Such a virus has predicted selective cytotoxicity to cells with inactivating mutations in the p53 pathway.

VI. References:

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VII. Appendix Contents:

1. Hoffman, W.H., Biade, S., Zilfou, J.T., Chen, J., Murphy, M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J. Biol. Chem. 277:3247-3257, 2002.

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Transcriptional Repression of the Anti-apoptotic *survivin* Gene by Wild Type p53*

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Survivin is a member of the inhibitor of apoptosis family. This apoptosis inhibitor also has an evolutionarily conserved role as a mitotic spindle checkpoint protein. Previous studies on p53-repressed genes have implicated several genes involved in the G_2/M transition of the cell cycle as targets of negative regulation by p53. However, few targets of p53 repression that are anti-apoptotic have been identified. This study identifies the anti-apoptotic *survivin* gene as a p53-repressed gene. Notably, Survivin repression by p53 is shown to be distinct from p53-dependent growth arrest. Chromatin immunoprecipitations indicate that p53 binds the *survivin* promoter *in vivo*; immunobinding studies indicate that this site overlaps with a binding site for E2F transcription factors and is subtly distinct from a canonical p53-transactivating element. The *survivin*-binding site contains a 3-nucleotide spacer between the two decamer "half-sites" of the p53 consensus element; deletion of this spacer is sufficient to convert the *survivin* site into a transactivating element. Finally, we show that overexpression of Survivin in cells sensitive to p53-dependent cell death markedly inhibits apoptosis induced by ultraviolet light. The identification of *survivin* as a p53 repressed gene should aid in the elucidation of the contribution of transcriptional repression to p53-dependent apoptosis.

survivin was first identified as a gene with a coding region complementary to the effector cell protease receptor, EPR-1. Although these genes share homology in their coding regions, they are transcribed in a reverse orientation and appear to share no regulatory or promoter regions (1). Sequence analysis of the *survivin* coding region revealed a conserved motif present at the amino terminus that identified it as a member of the inhibitor of apoptosis (IAP)[1] family. This motif, or baculovirus IAP repeat domain, has been shown to mediate the interaction with, and inhibition of, the caspase family of proteolytic enzymes, which are the penultimate mediators of apoptosis (2, 3). Consistent with its predicted anti-apoptotic function, expression of antisense RNA for Survivin is sufficient to induce apoptosis in a number of human tumor cell lines (4–6). Elevated expression of Survivin would be predicted to promote tumorigenesis, and in fact Survivin is highly expressed in a number of tumor types, including neuroblastoma, colorectal carcinoma, and gastric carcinoma; in these tumors, Survivin overexpression is correlated with poor prognosis (7–9). Additionally, analysis of the differences in gene expression between normal and tumor cells has revealed that *survivin* is one of the genes most consistently overexpressed in tumor cells relative to normal tissue (10).

Survivin is expressed widely in fetal tissues, but becomes restricted during development, and appears to be negligibly expressed in the majority of adult tissues (1, 11). The expression of Survivin is also cell cycle-regulated. This gene is repressed in the G_1 phase of the cell cycle and is highly expressed in G_2/M (12). This cell cycle regulation appears to rely on the presence of two CDE elements (cell cycle-dependent elements) that are downstream of the transcriptional start site (12), although a proximal CHR element (cyclin homology region) may also play a role. During mitosis, Survivin protein binds the mitotic spindle, and there is evidence that via this interaction this protein monitors mitotic spindle integrity; it is hypothesized that Survivin controls the elimination by apoptosis of those cells with aberrantly formed mitotic spindles (5, 13). This role for Survivin is evolutionarily conserved, as it is shared among homologues in yeast (14) and *Caenorhabditis elegans* (15). Although deregulation of *survivin* gene expression appears to be a common and significant event in tumorigenesis, little is known regarding the important regulators of the expression of this gene in normal and tumor cells.

Like Survivin, the p53 tumor suppressor protein is also a critical mediator of apoptosis and tumorigenesis. p53 is a nuclear transcription factor that is latent in normal cells but becomes activated by a variety of cellular stresses such as DNA damage, hypoxia (insufficient oxygen), and the presence of activated oncogenes (for review see Ref. 16). Following induction of p53 by these stresses, p53 up-regulates a set of genes that can promote cell death and growth arrest, such as $p21^{waf1}$, *bax*, *fas*, and *KILLER/DR5* (for review see Ref. 17). p53 also negatively regulates the expression of a separate set of genes; in some cases this negative regulation has been shown to be important for the induction of apoptosis (18–20). The exact nature of the binding site for p53 in repressed promoters, and how this site differs from that in transactivated promoters, has only begun to be elucidated. The mechanism of repression by p53 is also becoming more clearly elucidated; specifically, at least one mechanism whereby p53 negatively regulates gene expression involves an association between p53 and histone deacetylases (HDACs). This p53-HDAC interaction is mediated by binding of p53 to the co-repressor protein Sin3. The p53-Sin3 interaction targets HDACs to the promoters of p53-repressed genes, where HDACs serve to deacetylate histones and

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[1] The abbreviations used are: IAP, inhibitor of apoptosis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CDE, cell cycle-dependent elements; HDAC, histone deacetylase; Ab, antibody; PARP, poly(ADP) ribose polymerase; CMV, cytomegalovirus.

create a chromatin environment that is unfavorable for transcription (21).

In several studies the transcriptional repression activity of p53 has been implicated in p53-dependent apoptosis; notable in these studies is the finding that deletion of the proline-rich domain of p53 renders this protein competent as a transactivator but unable to induce apoptosis or to repress transcription (22–24). Additionally, tumor-derived mutant forms of p53 that are impaired for apoptosis induction are likewise unable to repress transcription, yet retain the ability to activate transcription (25). In support of the positive association between p53-mediated repression and apoptosis, we recently mapped the Sin3-binding domain of p53 to the proline-rich domain, which is critical for apoptosis induction by p53 (26). These and other studies raise the possibility that p53 may transcriptionally repress genes with anti-apoptotic activity. In a search for genes that are negatively regulated by p53, we and others identified several genes with roles in the control of the G_2/M phase of the cell cycle that are repressed following induction of wild type p53. These genes include *stathmin*, *Map4*, *cyclin B1*, *cdc2*, and *cdc25c* (27–30). Repression of these genes following DNA damage has been shown to require wild type p53 and is hypothesized to constitute a DNA damage-induced G_2/M checkpoint (31, 32). These studies prompted us to test the possibility that Survivin, which binds to the mitotic spindle and exhibits anti-apoptotic activity, might likewise be subject to negative regulation by p53. Our studies have identified *survivin* as a gene that is potently repressed, at both the RNA and protein levels, following p53 induction in cells with both endogenous and inducible p53. The identification of *survivin* as a p53-repressed gene should aid in the elucidation of the mechanism of transcriptional repression by p53 and in the estimation of the contribution of this activity to p53-dependent programmed cell death.

EXPERIMENTAL PROCEDURES

Cell Culture, p53 Induction—MCF-7 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin. The human melanoma cell line CaCl and the derivative clone expressing the *HPV E6* gene were cultured as described (27). The human osteosarcoma cell lines Saos2 and U2OS (kindly provided by Peter Adams, Fox Chase Cancer Center), the immortalized murine p21 knock-out fibroblasts (WD-50-5 cells, kindly provided by James Sherley, MIT, and established from cultures derived from Tyler Jacks, MIT), and the human lung adenocarcinoma cell line H1299 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin. Murine Val5 cells, containing the temperature-sensitive valine 135 allele of p53, human Val138 cells containing the valine 138 allele, and human tsp73 cells were maintained as described (33, 34). Unless otherwise noted all cells were grown at 37 °C in a 5% CO_2-humidified atmosphere. For treatment with ultraviolet light, cells were irradiated with 0–10 J/m2 (as noted) of UV-C with a Spectroline X series ultraviolet lamp, and output was monitored with a traceable UV light meter (Fisher). For drug treatment, cells were treated with 0.5 μg/ml adriamycin (doxorubicin, Sigma) or dilution vehicle alone for 24 h. p53 induction was monitored by Western analysis of 100 μg of protein as described (21), using 0.1 μg/ml DO-1 (Ab-6, Calbiochem) or 421 (Ab-1, Calbiochem).

Reverse Transcription-PCR, Northern Blots—The full-length coding region for Survivin was generated by reverse transcription-polymerase chain reaction using 5 μg of total RNA from the H1299 human lung adenocarcinoma cell line, using the Access Reverse Transcription-PCR kit (Promega) and the following oligonucleotides: forward 5′-ATGAGATACCATGGGTGCCCCGACG-3′, reverse 5′-TTAAGGATCCCTGCTCGATGGCACG-3′. The cDNA was cloned into the pCR3.1 vector (Invitrogen) and subjected to DNA sequence analysis for sequence confirmation. Total RNA was isolated from cells using CsCl purification (19) or using Trizol, as per the manufacturer (Invitrogen). Northern analyses were performed as described (19). Probes for Northerns were radiolabeled using random primers (Prime-It-II, Stratagene) and [α-^{32}P]dCTP (PerkinElmer Life Sciences). Autoradiographs were quantitated using NIH image software.

SDS-PAGE and Western Analysis—Western analysis was performed essentially as described (21). Briefly, subconfluent cells were harvested and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Protein concentrations were determined using the Bio-Rad D_C Protein assay (Bio-Rad). Equal amounts of protein (between 50 and 120 μg) were run on 10 or 12% SDS-polyacrylamide gels and transferred overnight onto polyvinylidene difluoride membrane (Bio-Rad). Western blots were incubated in antibody in 5% nonfat dry milk in phosphate-buffered saline supplemented with 0.2% Tween 20 (PBST). Blots were incubated with primary antibody at the following dilutions: p53 Ab-6 (Calbiochem) 1:1000, actin, AC-15 (Sigma) at 1:5000, $p21^{waf1}$ (Calbiochem) 1:1000, and Survivin (Oncogene Science) 1:1000. Blots were washed with PBST and incubated in horseradish peroxidase-linked secondary antibody (Jackson ImmunoResearch) and developed via the chemiluminescence protocol provided by the manufacturer (PerkinElmer Life Sciences).

PCR of the survivin Promoter, Generation of Deletion Constructs—Approximately 1.1 kb of the *survivin* promoter region was generated by polymerase chain reaction and cloned into the vector pCR 2.1 (Invitrogen) using the following oligonucleotides: forward 5′-CTGGCCATAGAACCAGAGAAGTGA-3′, reverse 5′-CCACCTCTGCCAACGGGTCCCGCG-3′, to generate the plasmid SpI. This sequence represents nucleotides 1821–2912 of the human *survivin* gene, GenBank™ accession number U75285. DNA sequence analysis confirmed the sequence of this region. The *survivin* promoter was then cloned into the promoterless luciferase vector pGL2-basic (Promega) to generate the plasmid SpII, for use in transfections and luciferase assays. The SpV deletion construct was generated from the SpI plasmid following digestion with *Sma*I and *Hin*dIII, generation of blunt ends by Klenow fill-in, and re-ligated; this promoter construct contains nucleotides 2331–2912 of the human *survivin* gene/promoter. For deletion of the p53-binding site in the *survivin* promoter, the SpV plasmid was digested with *Sac*II, which flanks the p53-binding site. Digestion of SpV with *Sac*II and religation of this construct eliminates the p53-binding site, as well as approximately 30 nucleotides upstream of the site, to generate the construct SpV-Δp53BS. The Sp-min construct consists of the p53-binding site and the adjacent E2F-binding site; this construct was generated using PCR using the following overlapping oligonucleotides: 5′-AAAGGTACCGGGCGTGCGCTCCCGACATG-3′ and 5′-AAACTCGAGGGCGCGCCGCGGGGCATGTCG-3′. This fragment was cloned into the plasmid pGL3-E1B-TATA, kindly provided by James Manfredi (Mount Sinai School of Medicine). The SpVII construct was generated in an identical manner, except that oligonucleotides were designed that deleted the 3-nucleotide spacer in the p53-binding site.

Transfections, Luciferase Assays—H1299, SaoS-2, or WD-50-5 (p21 −/− cells) cells were seeded in 10-cm plates at 1×10^6 cells/plate and allowed to settle overnight. The next morning cells were transfected with 4 μg of firefly luciferase reporter construct and 2–4 μg of *Renilla* luciferase construct (pRL-CMV or pRL-tk, Promega) or SV40 β-galactosidase, along with the indicated amounts of p53 expression plasmid (in pRc/CMV) using Fugene, according to protocols derived from the manufacturer (Roche Molecular Biochemicals). After 24 h, the cells were harvested and lysed, and dual luciferase assays were performed as per the protocol derived from the manufacturer (Promega) on a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Luciferase activity was normalized to total protein levels, as well as to *Renilla* luciferase activity or β-galactosidase activity; β-galactosidase assays were performed according to protocols derived from the manufacturer (Promega).

Kinase Assays, Flow Cytometry—Cyclin-dependent kinase assays were performed essentially as described (35), using 250 μg of whole cell extract immunoprecipitated with 0.5 μg of polyclonal antisera to Cdk2 (Santa Cruz Biotechnology), cyclin E, and cyclin A. Following immunoprecipitation and washing, 1 μg of histone H1 (Sigma) was used as a substrate in kinase reaction buffer (20 mM Tris, pH 7.4, 7.5 mM $MgCl_2$, 1 mM dithiothreitol) supplemented with 5 mM sodium fluoride, 1 mM sodium 32 °C orthovanadate, and 125 ng/μl cAMP-dependent protein kinase inhibitor (Sigma). 0.5 μl of [γ-^{32}P]ATP (PerkinElmer Life Sciences, 800 Ci/mmol) was added to each reaction, and 0.5 μg of normal rabbit IgG (Sigma) was used as a negative control. Following incubation at 37 °C for 20 min, reactions were boiled in Laemmli sample buffer and loaded onto 10% SDS-PAGE. Cells for flow cytometry were fixed in ethanol and stained with propidium iodide as described (19) and analyzed on a Becton Dickinson FacScan. G_1, S (synthesis), and G_2/M populations were calculated using the program CellQuest.

Modified McKay Assays (Immunobinding), Chromatin Immunopre-

cipitations—Immunoselection of the p53-binding site in the *survivin* promoter was performed essentially as described (33, 36). Briefly, 100–200 μg of whole cell extract from 10.1 (p53 null) or Val5 cells grown at (wt p53) was incubated with 300,000 cpm of end-labeled probe for the *survivin* promoter in McKay binding buffer (10% glycerol, 5 mM EDTA, 20 mM Tris, pH 7.2, 100 mM NaCl, 0.1% Nonidet P-40) supplemented with 1.25 μg/μl poly(dI/dC) (Amersham Biosciences) and 1 μg each 421 and 1620 monoclonal antibody. Following incubation for 1 h at 4 °C, immune complexes were collected with protein A-Sepharose and washed extensively in McKay washing buffer (2% glycerol, 5 mM EDTA, 20 mM Tris, pH 7.2, 100 mM NaCl, 0.1% Nonidet P-40), resuspended in 10 mM Tris, pH 7.4, 1 mM EDTA, pH 8, phenol/chloroform extracted, precipitated, and resolved on 4% non-denaturing acrylamide gels. For competition experiments, a 20 5-fold molar excess of unlabeled DNA was preincubated with extract for 10 min prior to the binding reaction. Chromatin immunoprecipitations were performed exactly as described (21) using primers specific for the *survivin* promoter and polyclonal antisera specific for p53 (Santa Cruz Biotechnology) and acetylated histone H3 (Upstate Biotechnology, Inc.). To ensure for linearity, PCRs were sampled and analyzed following 25, 30, and 35 cycles.

Site-directed Mutagenesis, Generation of Stable Cell Lines Overexpressing Survivin—The Survivin cDNA was mutagenized to convert it to a constitutively active anti-apoptotic form by changing the threonine residue at amino acid 34 to glutamic acid (T34E), using the QuikChange site-directed mutagenesis protocol (Stratagene). This mutant, which had significantly less toxicity than the wild type cDNA, was cloned into a CMV-driven plasmid (CMV-neo-Bam3) and transfected into CaCl cells. Subclones were isolated and analyzed for apoptosis induction following treatment with ultraviolet radiation using annexin V assays (R & D Systems), as well as immunofluorescence and Western analysis using the p85 PARP antibody, which is specific for the caspase-cleaved form of PARP, according to protocols derived from the supplier (Promega).

RESULTS

Survivin Is Negatively Regulated in a p53-dependent Manner—Previous studies using DNA micro-array technology to identify candidate p53-repressed genes revealed that a number of genes with roles in G_2/M progression, including several genes that encode microtubule components, are subject to negative regulation by p53 (30). We sought to test the hypothesis that, like these genes, the microtubule-associated protein Survivin might likewise be negatively regulated by p53. For these studies the human lung adenocarcinoma cell line Val138 was utilized; these cells are derived from p53-null H1299 cells and are stably transfected with a temperature-sensitive p53 allele encoding valine at codon 138. The p53 protein in these cells exists in a mutant conformation at 39 °C, and temperature shift to 32 °C induces a wild type conformation of p53 and concomitant growth arrest (34). A cDNA clone for Survivin, generated by reverse transcription-PCR (see "Experimental Procedures") was utilized as a probe in Northern analyses of Val138 cells cultured at 39 °C (mutant p53) or temperature-shifted to 32 °C (wt p53) for 24 h. As shown in the Northern analysis in Fig. 1*A*, temperature shift of parental H1299 cells had no effect on Survivin RNA levels (*lanes 1* and *2*). However, temperature shift in Val138 cells resulted in a significant decrease in Survivin levels (*lanes 3* and *4*), whereas the levels of the housekeeping gene *gapdh* and the IAP gene *XIAP* remained unchanged (see Fig. 1).

In an effort to examine the effects of endogenous p53 induction on *survivin* gene expression, human tumor cell lines containing wild type p53 were treated with the DNA-damaging agent doxorubicin (adriamycin), which is a potent inducer of p53 protein and p53-dependent transcriptional activity. Treatment of the human MCF-7 breast carcinoma and the human melanoma cell line CaCl with doxorubicin led to significant decreases in the steady state level of Survivin messenger RNA as early as 12 h after treatment (see Fig. 1*B*). Similarly, treatment of the human osteosarcoma cell line U2OS (wt p53) with doxorubicin led to significant induction of p53 and a 5-fold decrease in Survivin protein after 24 h (Fig. 1*C*). In contrast,

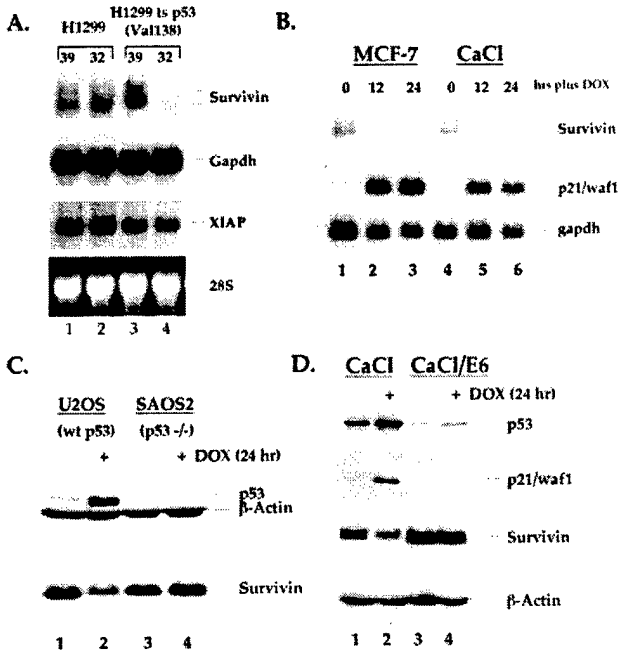


FIG. 1. **Survivin is down-regulated following induction of wild type (wt) p53.** *A*, Northern analysis of *survivin* gene expression in cells containing no p53 (human H1299 lung adenocarcinoma) and temperature-sensitive p53 (Val138, wt p53 at 32 °C). Cells in *lanes 2* and *4* were temperature-shifted to 32 °C (wt p53) for 24 h. As loading controls, the levels of the housekeeping gene *gapdh*, and the inhibitor of apoptosis XIAP, are depicted. *B*, Survivin mRNA is down-regulated following p53 induction in cells containing wt p53. Induction of wt p53 in human breast carcinoma cells (MCF-7) and melanoma cells (CaCl) following doxorubicin (*DOX*) treatment results in marked decreases in mRNA levels for Survivin, as early as 12 h following treatment (0.5 μg/ml doxorubicin). In contrast, doxorubicin treatment results in increased expression of the p53-induced gene $p21^{waf1}$, whereas the RNA levels of GAPDH remain unchanged. *C*, Survivin down-regulation requires the presence of wt p53. Western analysis of the human osteosarcoma cell lines U2OS (wt p53) and Saos2 (p53-null) following doxorubicin treatment results in decreased Survivin protein levels only in cells with wt p53 (U2OS). A β-actin control is included to verify equal protein loading in the lanes. *D*, the human papillomavirus E6 protein, which targets p53 for degradation, inhibits doxorubicin-mediated down-regulation of *survivin* by p53. Western analysis of the human melanoma cell line CaCl and a clonal derivative of this cell line stably express the human papillomavirus E6 protein (CaCl/E6) following treatment with the DNA-damaging agent doxorubicin (*DOX*) for 24 h. Doxorubicin treatment results in down-regulation of Survivin protein levels only in parental CaCl cells, which contain functional p53. As a positive control for p53 induction, immunoblots of p53 and of the p53-induced gene $p21^{waf1}$ are included. A β-actin control is included to verify equal protein loading in the lanes.

such decreased levels did not occur following identical treatment of Saos2 human osteosarcoma cells, which are null for p53 (Fig. 1*C*, *lane 4*). To extend these studies, we performed Western analysis of p53 and Survivin in matched human tumor cell lines that differ in p53 status, following treatment with doxorubicin. CaCl is a human melanoma cell line with wt p53; CaCl/E6 is a clonal derivative of this cell line that stably expresses the human papillomavirus protein E6, which targets p53 for degradation (27). As shown in Fig. 1*D*, the CaCl/E6 line has significantly reduced p53 levels compared with parental CaCl cells, and doxorubicin treatment failed to up-regulate the p53-induced protein $p21^{waf1}$. Similarly, doxorubicin treatment failed to cause down-regulation of Survivin in CaCl/E6 cells, indicating a requirement for wt p53 for this process. Flow cytometric analyses indicated that both cell lines undergo G_2/M arrest following doxorubicin treatment, indicating that Survivin down-regulation was unlikely to be an indirect effect of p53-induced G_1 arrest in these cells (see below).

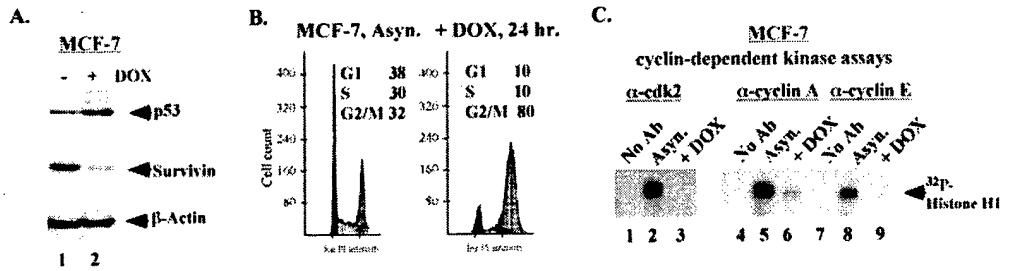


FIG. 2. **p53 induction in MCF-7 cells leads to *survivin* down-regulation and G_2/M arrest.** *A*, Western analysis of p53 induction in human MCF7 breast carcinoma cells, following 24 h treatment with doxorubicin which creates DNA damage and induces p53. Although p53 levels are 4-fold induced, protein levels of Survivin are 3–4-fold decreased. A β-actin control is included to verify equal protein loading in the lanes. *B*, flow cytometry profiles of MCF-7 cells treated with doxorubicin (*+DOX*) for 24 h. Propidium iodide staining indicates that MCF-7 cells arrest with a 4N DNA content; the percentage of cells in each cell cycle stage are indicated. The data depicted are representative of three independent experiments. *C*, immunoprecipitation-kinase assays of catalytically active Cdk2 (*lanes 2* and *3*), cyclin A (*lanes 5* and *6*), and cyclin E (*lanes 8* and *9*) using histone H1 as the substrate. As a negative control, normal rabbit IgG is used instead of polyclonal antisera for the immunoprecipitations and kinase assays (*No Ab*, *lanes 1, 4,* and *7*). Notably, whereas these kinase activities are high in asynchronously growing MCF7 cells (*Asyn*, *lanes 2, 5,* and *8*), all activities are low in cells treated with doxorubicin (*lanes 3, 6,* and *9*). This finding is consistent with flow cytometry data, which indicate that doxorubicin-treated cells are arrested with a G_2/M content of DNA.

p53-dependent Repression of survivin Occurs Independent of G_1 Arrest by p53—Because the *survivin* gene is known to be transcriptionally repressed during the G_1 phase of the cell cycle, it became important to eliminate the possibility that repression of Survivin by p53 was a side effect of the induction of G_1 growth arrest by this protein. Immunoblots of MCF-7 cells treated with doxorubicin indicated that, as expected, p53 levels were induced, and Survivin levels were markedly decreased following treatment (Fig. 2*A*). Flow cytometric analysis of these cells indicated that they were growth-arrested in the G_2/M phase of the cell cycle following doxorubicin treatment (Fig. 2*B*); such a failure of human tumor cell lines with wt p53 to growth arrest in G_1 following doxorubicin and gamma irradiation treatment has been noted by others (37, 38). In an effort to confirm that these doxorubicin-treated cells were in fact arrested in G_2/M, we performed cyclin-dependent kinase assays in MCF-7 cells before and after drug treatment. These assays indicated that the activity of the G_1-specific cyclin E-associated kinase, as well as the Cdk2 kinase, were dramatically reduced in MCF-7 cells following doxorubicin treatment (Fig. 2*C, lanes 3* and *9*). Additionally, the kinase activity associated with cyclin A, the S phase cyclin, was also markedly reduced (Fig. 2*C, lane 6*), as would be expected for cells in G_2/M. Because the *cyclin B1* and *cdc2* genes are subject to transcriptional repression by p53, the activity of these enzymes was not analyzed; however, the combined data support the premise that doxorubicin-treated MCF7 cells are arrested in G_2/M.

We extended these studies in efforts to better separate the ability of p53 to down-regulate Survivin from its ability to induce G_1 arrest. We (27) and others (39) have noted that treatment of cells with low doses of UV radiation, between 2 and 4 J/m^2, is sufficient to induce p53 protein and transcriptional activity but is insufficient to induce G_1 growth arrest. Treatment of MCF-7 breast carcinoma cells with ultraviolet irradiation led to dose-dependent decreases of Survivin protein 24 h after treatment, whereas flow cytometry indicated that these cells continue to proliferate following treatment (Fig. 3*A* and data not shown). We also analyzed murine embryo fibroblasts from the genetically engineered p21^{waf1} knock-out mouse; these cells are markedly impaired for growth arrest in response to p53 (40). p21-null mouse embryo fibroblasts demonstrated normal p53 induction and decreased Survivin levels, following treatment with UV radiation (Fig. 3*B*), but there was no evidence for G_1 arrest by flow cytometry (Fig. 3*C*). These data solidify the notion that the ability of p53 to down-regulate Survivin is distinct from its ability to induce G_1 arrest or to induce p21^{waf1}.

It became of interest to test the ability of the p53 homologue p73 to down-regulate the *survivin* gene; although p73 is fully capable of inducing p21^{waf1} and G_1 arrest, it has been reported to be incapable of repressing the p53-repressed gene *cdc25c* (41). For this analysis we took advantage of p53-null H1299 cell lines that have been stably transfected with temperature-sensitive versions of p53 and p73 protein (34). As indicated previously, these proteins exist in mutant (inactive) conformation at 39 °C and become wild type conformation (and activity) at 32 °C. As depicted in Fig. 4, only temperature-sensitive p53, and not p73, was capable of down-regulating Survivin at the permissive temperature (32 °C, wt p53, *lane 4*). In contrast, both proteins were able to transactivate p21^{waf1} to identical levels (Fig. 4*A*), and both were indistinguishable in their ability to growth-arrest cells (Fig. 4*B*). The combined data indicate that induction of growth arrest and transactivation of p21^{waf1} are not sufficient to cause down-regulation of Survivin. In efforts to address whether p53 directly negatively regulates the *survivin* promoter, we cloned a 1-kb fragment of this promoter, and we analyzed it for the ability to confer negative regulation to a reporter gene by p53.

The survivin Promoter Is Sufficient to Confer Negative Regulation to a Heterologous Gene by p53, Even in Stably Transfected Cells—The *survivin* promoter has been characterized previously (5, 12). We cloned ~1 kb of this promoter by PCR of human genomic DNA using primers designed from the published sequence (see "Experimental Procedures"). The full-length 1-kb *survivin* promoter was cloned upstream of the firefly luciferase reporter gene to create the reporter construct SpII. To test the repressibility of this promoter by p53, this construct was transfected into p53 null cells (human H1299 cells) with increasing concentrations of p53. As a control for the ability of p53 to nonspecifically repress transcription (so-called "transcriptional squelching"), equal microgram amounts of the *Renilla* luciferase gene were transfected, and the ratio of firefly to *Renilla* luciferase per equal microgram amount of protein was determined following transfection using standard assays. As depicted in Fig. 5A, as little as 10 ng of wt p53 was sufficient to repress 5 μg of the *survivin* promoter, indicating that this promoter is quite sensitive to p53-dependent repression. Increasing concentrations of p53 led to stepwise decreases in the activity of the luciferase gene driven by the *survivin* promoter (Fig. 5*A*). Deletion analysis of this promoter indicated that a truncated version (encoding nucleotides −105 to −15, where +1 denotes the initiating ATG, as per 5) was also negatively regulated by p53. This construct, SpVI, demonstrated a lower basal level of transcription than the full-length *survivin* pro-

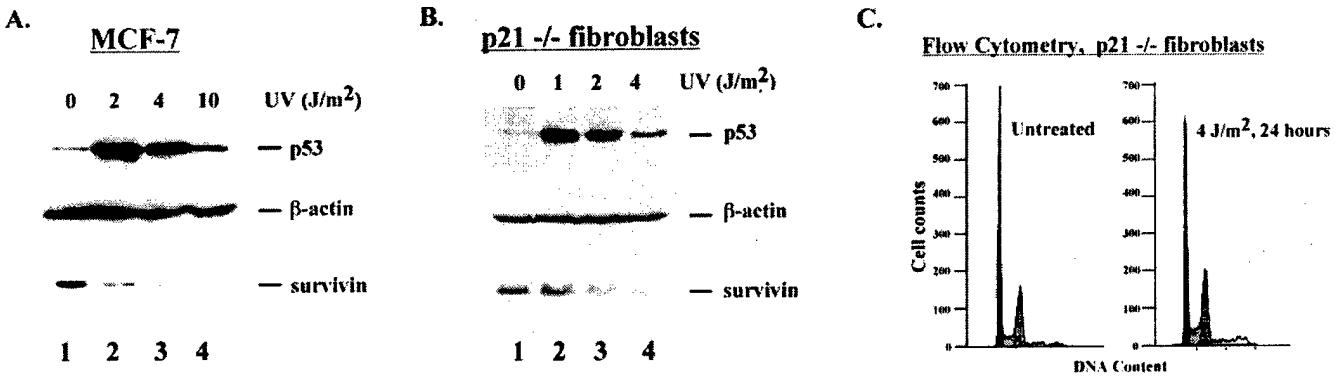
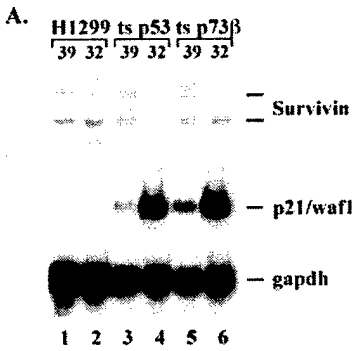


FIG. 3. **Down-regulation of *survivin* by p53 does not require induction of G_1 arrest or $p21^{waf1}$.** *A*, dose-dependent decreases in *survivin* gene expression in MCF7 breast carcinoma cells following ultraviolet irradiation. Cells were treated with the indicated dose of radiation, harvested 24 h later, and subjected to Western analysis for p53 and Survivin. That there is less p53 in the 10 J/m² sample (*lane 4*) reflects the fact p53 has already peaked in level in these cells and that levels of p53 are returning to normal. A β-actin control is included to verify equal protein loading in the lanes. *B*, Western analysis of murine embryo fibroblasts from the $p21^{waf1}$ knock-out mouse reveals dose-dependent decreases in Survivin levels in these cells following ultraviolet irradiation. A β-actin control is included to verify equal protein loading in the lanes. *C*, flow cytometric analyses of $p21^{waf1}$-null mouse embryo fibroblasts that are treated with ultraviolet radiation (4 J/m²) or untreated. The calculated numbers indicate there is no evidence for G_1 arrest following p53 induction in this cell line; the data depicted are representative from two independent experiments read in duplicate.



B.

Flow cytometry Data

	39			32 (wild type)		
	G1	S	G2/M	G1	S	G2/M
H1299 ts p53 (Val138)	46	34	20	62	8	30
H1299 ts p73b	42	27	31	68	8	24

FIG. 4. **Inducible p53, but not p73β, negatively regulates *survivin* gene expression.** *A*, Northern analysis of Survivin levels in H1299 cells (p53-null human lung adenocarcinoma, *lanes 1* and *2*), and in H1299 cells containing stably transfected alleles for temperature-sensitive p53 (*ts p53* Val138 cells, *lanes 3* and *4*), and in p73β (*lanes 5* and *6*). Cells were grown at 39 °C (mutant conformation, *lanes 3* and *5*) or temperature-shifted to 32 °C for 24 h (wild type p53/p73 induction) as indicated. Whereas both proteins are capable of inducing the p53-response gene $p21^{waf1}$ to comparable levels at 32 °C (*lanes 4* and *6*), only wild type p53, and not p73β, was capable of down-regulating *survivin* expression (*lane 4*). Like p73β, p73α was likewise incapable of repressing *survivin* expression, despite being capable of inducing growth arrest in temperature-shifted cells (not shown). A GAPDH control is included to verify equal loading and integrity of RNA. The data depicted are representative of three independent experiments. *B*, flow cytometric analysis of temperature-shifted, propidium iodide-stained cells containing temperature-sensitive p53 (*ts p53* Val138 cells) or p73β. Both proteins are capable of inducing a G_1 and G_2/M arrest following 24-h temperature shift to 32 °C, whereas only ts p53 can repress *survivin* (*A*). The data presented are the average from two independent experiments, and the same plates of cells were used for the Northern analyses in *A*.

moter, but was repressed nearly as well by wt p53 (Fig. 5*A*). Similar results were obtained in the human Saos2 osteosarcoma cell line (Fig. 5*B*); this cell line is null for both p53 and the retinoblastoma protein pRB and thus is impaired for p53-dependent growth arrest. These data also support the conclusion that p53 can down-regulate Survivin independent of p53-dependent growth arrest.

Repression of Survivin by p53 was recapitulated in stably transfected cells, where the full-length *survivin* promoter-luciferase construct was stably introduced into cells containing temperature-sensitive p53 (Val5-SpII cells). In two independently derived cell lines containing temperature-sensitive p53, the stably integrated luciferase gene driven by the *survivin* promoter was markedly down-regulated by p53 induction (Fig. 5*C*). Similarly, we found that the luciferase construct containing a truncated version of the *survivin* promoter (SpVI) was also sufficient to confer negative regulation by p53 in stably transfected cells (data not shown). In contrast, temperature shift alone had no effect on luciferase levels in parental 10.1 mouse embryo fibroblasts (data not shown). To our knowledge, the *survivin* promoter is the first promoter demonstrated to confer p53-dependent repression to a heterologous gene in both transiently and stably transfected cells.

p53 Binds in a Sequence-specific Manner to the survivin Promoter; the p53-binding Site Is Necessary for Transcriptional Repression of Survivin in Vivo—To test whether p53 could physically associate with the *survivin* promoter, the assay of McKay was utilized; this assay allows for the analysis of DNA-protein interactions using large fragments of DNA, up to several kilobases in length (36). In this assay, the *survivin* promoter was digested with restriction endonucleases, radiolabeled with [^{32}P]dCTP using Klenow polymerase, and incubated with whole cell extract from cells that are null for p53 (10.1 murine embryo fibroblasts) or that contain wild type p53 (Val5 cells shifted to 32 °C). Following incubation of the radiolabeled *survivin* promoter with cell extract, samples were immunoprecipitated with p53 antisera and protein A-Sepharose. These immunoprecipitates were washed and phenol/chloroform extracted, and bound DNA fragments were resolved on non-denaturing polyacrylamide gels. McKay assays on the full-length *survivin* promoter (SpII, not shown), and on the smaller SpV fragment of this promoter (Fig. 6*A*), indicated that this

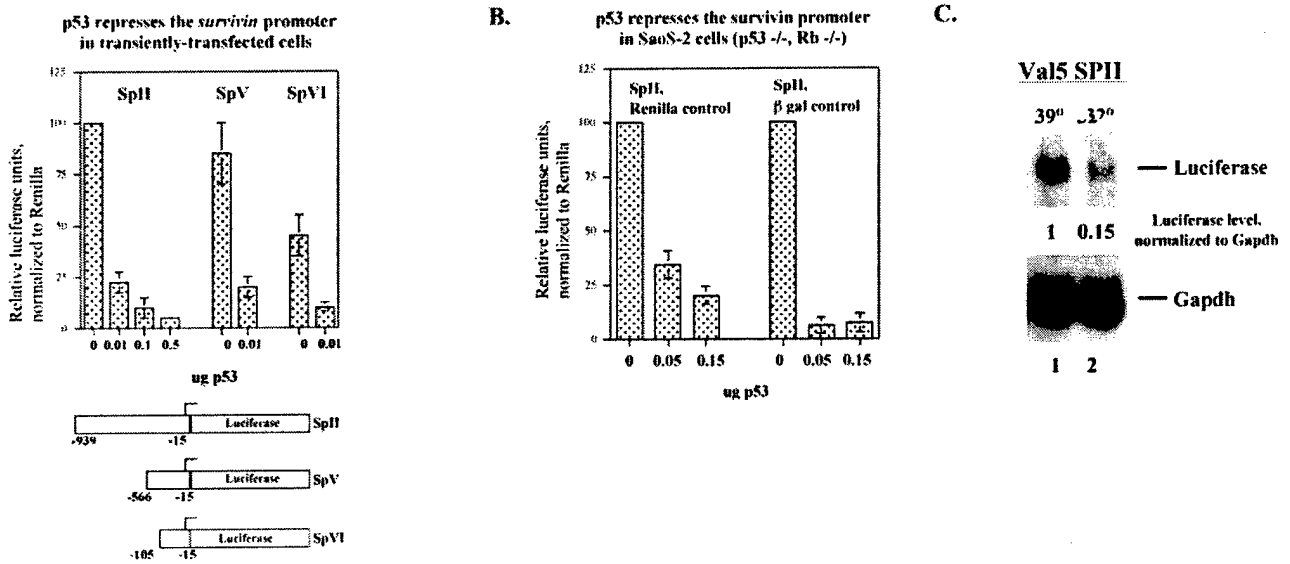


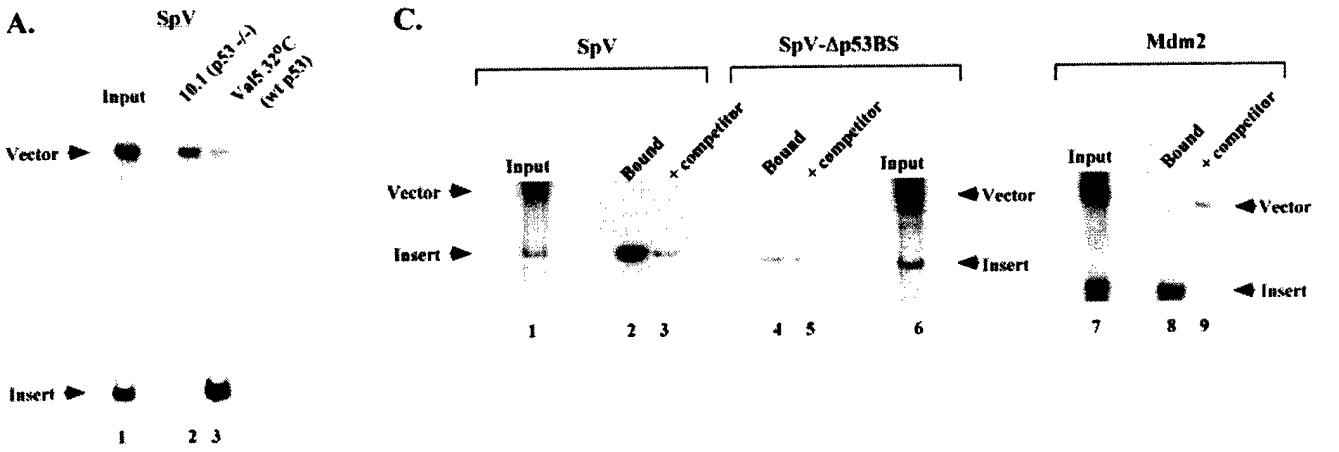
FIG. 5. *A*, the *survivin* promoter is sufficient to confer negative regulation of the firefly luciferase gene by p53. The *survivin* promoter, ligated to the promoterless firefly luciferase gene (pGL2-basic, Promega) to create the construct SpII, was transiently transfected with increasing concentrations of wild type p53 (0, 0.01, 0.1, and 1 μg of p53). Deletion constructs of the *survivin* promoter (SpV and SpVI, depicted to the *right* and numbered as per Ref. 5, where +1 represents the ATG), which contain the nucleotides depicted, were separately transfected with 10 ng of p53. As a control for transfection efficiency and nonspecific repression, equal micrograms of Sp construct and CMV-*Renilla* luciferase were transfected with the p53 amounts indicated, and the numbers shown are normalized to *Renilla* values using the Dual Luciferase assay (Promega) and to protein levels using the Bio-Rad Dc assay (Bio-Rad); the level of SpII was set at 100%. The results depicted are the average of three independent experiments; *error bars* mark S.D. *B*, the *survivin* promoter is negatively regulated by p53 in cells that are incapable of G_1 arrest. Human Saos-2 cells (p53 −/−, pRB −/−) were transiently transfected with 4 μg of SpII (full-length *survivin* promoter driving firefly luciferase expression) and increasing doses of p53. The numbers shown are normalized to equal protein levels (Bio-Rad Dc assay) and to the activity of equal microgram amounts of co-transfected *Renilla* luciferase (CMV-*Renilla*, Promega) or SV40-β-galactosidase (Promega). *C*, the full-length *survivin* promoter (SpII) is sufficient to confer negative regulation by p53 in stably transfected cells. The *survivin* construct SpII was co-transfected into cells containing temperature-sensitive p53 (Val5 cells) in 5-fold excess with the drug selectable plasmid pGK-hygro, which confers hygromycin resistance. Hygromycin-resistant colonies were pooled and analyzed. Temperature shift to 32 °C induces wt p53 protein and down-regulation of luciferase mRNA, as depicted in this Northern analysis. Expression of GAPDH is shown as a control for RNA loading and integrity. The results shown were recapitulated in three independent experiments, in two independent sets of pooled, stably transfected cells.

DNA fragment could be specifically immunoprecipitated with p53 antisera, only in cells containing wild type p53 (Fig. 6*A*, *lane 3*) and not in parental p53-null cells (*lane 2*). As an internal negative control, vector sequences were negligibly bound to p53 in this assay (Fig. 6*A*). The *survivin* promoter was capable of binding to p53 to a level roughly comparable with the *Mdm2* promoter, which was labeled to identical specific activity (Fig. 6*C*, *lane 8*).

The sequence of the minimal p53-binding region of the *survivin* promoter defined from these assays is depicted in Fig. 6*B*. Inspection of this region revealed a consensus p53-binding site that differs from a transactivated element in that it contains a 3-nucleotide spacer element between the two pairs of palindromic pentamers, or "half-sites" (Fig. 6*B*). To date, the majority of p53-transactivated genes contain a spacer of 0 or 1 nucleotide; in fact, lengthening this spacer to 4 nucleotides has been shown to abolish the ability of p53 to transactivate promoters containing this binding site (42). To test the possibility that this candidate-binding site was responsible for the *in vitro* binding we detected, this site was deleted by restriction endonuclease digestion from the *survivin* promoter and tested in a McKay assay. Deletion of this candidate p53-binding site from the *survivin* promoter was sufficient to completely abolish p53 binding in a McKay assay (Fig. 6*C*, *lane 4*). That binding of the *survivin* promoter by p53 was sequence-specific was supported by the finding that it could be eliminated by incubation with a 20 5-fold excess of unlabeled DNA (Fig. 6*C*, *lane 3*).

To assess the significance of the p53-binding site on transcriptional repression of *survivin* by p53, these same *survivin* promoter/luciferase constructs (SpV and SpVΔp53BS) were stably transfected into Val5 cells that contain a temperature-sensitive (inducible) p53. Significantly, whereas p53 negatively regulated the luciferase gene driven by the wild type *survivin* promoter (SpV, Fig. 6*D*), deletion of the p53-binding site effectively inhibited this repression (SpV-Δp53BS). Therefore, the p53-binding site of the *survivin* promoter is necessary for both binding and for transcriptional repression by p53.

Of interest in the *survivin* promoter is the observation that the p53-binding site overlaps with a site homologous to those utilized by E2F family members (2 mismatches). This prompted us to test the possibility that E2F family members could transactivate the *survivin* promoter and furthermore that p53 might repress *survivin* in part by interfering with E2F-mediated transactivation of this gene. To test these possibilities, a minimal *survivin* promoter construct, containing only the p53-binding site and the overlapping E2F site, was constructed in the plasmid pGL3-E1B-TATA, which contains the luciferase gene and the TATA box of the adenovirus *E1B* gene (see "Experimental Procedures"). This construct, designated Sp-min, was transfected into H1299 cells with increasing concentrations of p53 plasmid. As indicated in Fig. 7, when normalized to control and protein concentrations, the Sp-min construct was repressed by p53 in a dose-dependent manner (Fig. 7*A*). Additionally, transfection with E2F-1 was sufficient to activate transcription from this promoter; typically this induction ranged from 3- to 10-fold (Fig. 7*A*). Additionally, the SpII construct, containing the full-length *survivin* promoter, was also potently up-regulated by E2F-1 (data not shown). Notably, E2F-mediated activation of Sp-min was inhibited by increased concentrations of p53 (Fig. 7*A*). These data support the hypothesis that p53 may repress the *survivin* promoter in part by inhibiting the ability of E2F proteins to transactivate this gene. Interestingly, the ability of p53 to repress E2F-mediated transactivation of this construct did not rely on



B. Sequence of the survivin promoter

```
GCCTCTACTC CCAGAAGGCC  GCGGGGGGTG GACCGCCTAA
           p53         E2F-like    +1
GAGGGCGTGC GCTCCCGACA TGCCCCGCGG CGCGCCATTA
  CDE      CHR
ACCGCCAGAT TTGAATCGCG GGACCCGTGG GCAGAGGTGG

CGGCGGCGGC ATGGGTGCCC
```

D. Deletion of the p53-binding site in the survivin promoter abrogates transcriptional repression by p53 in vivo.

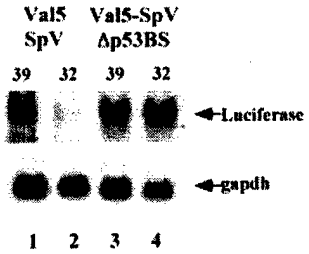


FIG. 6. **p53 binds in a sequence-specific manner to a fragment of the *survivin* promoter.** *A,* McKay immunobinding assays indicate that the radiolabeled *survivin* promoter (*SpV*), but not the vector internal control, is immunoprecipitated by p53 antisera in cells with wt p53 (Val5 32 °C, *lane 3*) but not in cells that are null for p53 (parental 10.1 cells, p53 −/−, *lane 2*). 20% of the radiolabeled vector/insert is loaded in the *input lane. B,* sequence of the *survivin* promoter in the minimal p53-binding region. The *arrow* denotes the start site of *survivin* transcription; just upstream of the transcriptional start site is depicted the p53-binding site (*boxed*) and an overlapping consensus for E2F transcription factors (*underlined*). Downstream elements responsible for preferential G_2/M transcription, the CDE/CHR, are *underlined. C,* immunobinding (McKay) assays using the radiolabeled promoter constructs listed, incubated with 100 μg of whole cell extract from cells with wt p53 and monoclonal antisera specific for p53. Following binding reactions, bound protein-DNA complexes are immunoprecipitated, washed, phenol/chloroform extracted, and resolved on a non-denaturing polyacrylamide gel. The radiolabeled vector fragment serves as an internal negative control and the *Mdm2* promoter serves as a positive control (*lanes 7–9*). The SpV construct encodes an ~500-bp fragment of the *survivin* promoter; the SpV-Δp53BS encodes the same fragment, with the p53-binding site deleted using the restriction endonuclease *Sac*II. As a control for sequence-specific binding, a 25-fold molar excess of each construct was preincubated with the extract for 10 min prior to the binding reaction (+ *competitor*). 10% of the radiolabeled vector/insert is loaded in the *input lane*. The results depicted are representative of several independent experiments. *D,* Northern analysis of cells with temperature-sensitive p53 (Val5 cells, murine p53-null cells transfected with the valine 135 temperature-sensitive p53 gene) that are stably transfected with the SpV-*survivin* promoter/luciferase construct or the SpV construct in which the p53-binding site is deleted (SpVΔp53BS). Northern hybridization to a probe specific for the luciferase gene indicates that p53 is capable of down-regulating the SpV construct but not the construct in which the p53-binding site is deleted (SpVΔp53BS). The data depicted are from pooled, stably transfected clones and are representative of three independent experiments on two independently derived sets of cell lines for each construct.

$p21^{waf1}$, as p53-mediated repression of the Sp-min construct was also evident in p21 −/− cells (Fig. 7*B*), albeit to a slightly reduced magnitude. The combined data raise the possibility that p53 may repress *survivin* transcription in two ways as follows: one may involve inhibition of E2F by induction of $p21^{waf1}$, and subsequent binding of hypophosphorylated pRB to E2F to create a transcriptional repressor. Additionally, however, p53 appears to interact directly with the *survivin* promoter and may interfere with the ability of E2F proteins from activating transcription from an overlapping E2F site.

p53 Interacts with the survivin Promoter in Vivo; Deletion of the Sin3 Binding Domain of p53 Impairs Its Ability to Repress Survivin—We have shown previously that one mechanism whereby p53 can repress gene expression is via binding to the co-repressor Sin3, which recruits HDACs to the promoters of p53-repressed genes like *Map4* (21). That p53 binds to the *Map4* promoter *in vivo*, and recruits HDACs, was demonstrated using the technique of chromatin immunoprecipitations. Chromatin immunoprecipitations were performed on the *survivin* promoter in cells containing wild type (Val5-SpII cells at 32 °C) and mutant (cells at 39 °C) p53. These data indicated that wt but not mutant p53 could be found complexed to the *survivin* promoter *in vivo* (Fig. 8*A*); this binding was comparable with that for the *Map4* promoter in these cells (Ref. 21 and data not shown). Additionally, in cells with wt p53 (cells at 32 °C), but not mutant p53 (39 °C), the *survivin* promoter was preferentially associated with deacetylated histone H3, consistent with the action of HDACs (Fig. 8*A*, *lanes 5* and *6*). Identical reactions performed in the absence of antibody, with irrelevant antibody, or in wash buffers alone, failed to reveal a PCR product for the *survivin* promoter (Fig. 8*A*, *lanes 1–3*). These data were consistent in three independent experiments, and they support our data from the McKay assay, which indicate that wild type p53 can interact with a consensus p53-binding site within the *survivin* promoter.

We recently mapped the interaction domain between Sin3 and p53, and we have found that amino acids 61–75 of wild type p53 are necessary for interaction with Sin3 (26). Therefore, it became of interest to determine whether a deletion mutant of p53 lacking the Sin3-binding domain (Δ61–75) was impaired for repression of the *survivin* promoter. p53-null H1299 cells were transfected with the luciferase construct SpVI, in the presence of 10 ng of either wt p53 or the Δ61–75 mutant of p53. Significantly, whereas wt p53 was capable of

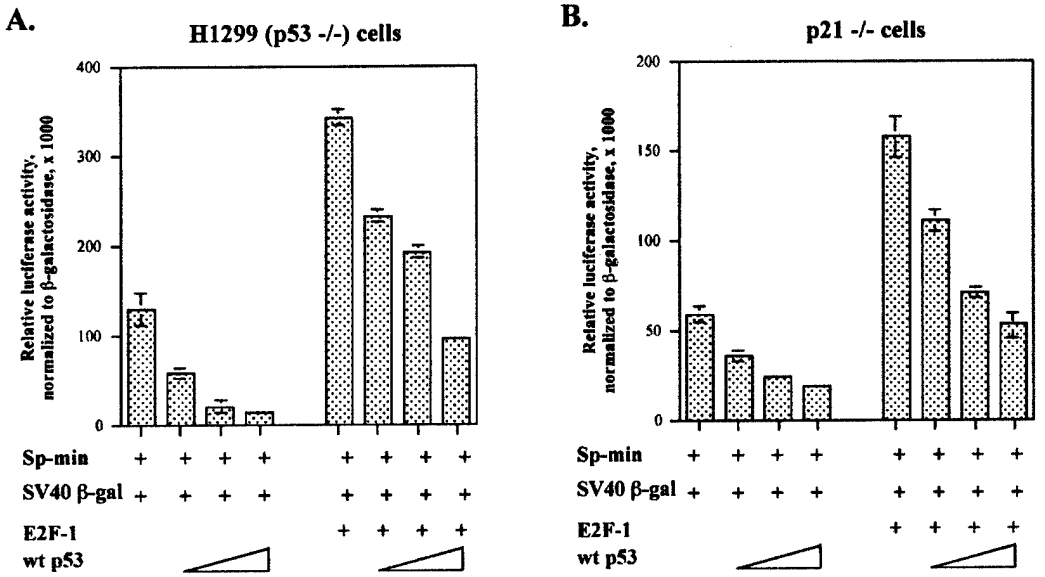


FIG. 7. **Increasing concentrations of p53 overcome E2F-mediated transactivation of the *survivin* promoter in p53 null (H1299) and p21-null cells.** Transient transfection/luciferase assays of 4 μg of the minimal p53-repressible *survivin* promoter (Sp-min, contains the p53-binding site and the overlapping E2F site, in the luciferase construct pGL3-E1B-TATA) were incubated with increasing concentrations of p53 (0, 0.01, 0.1, and 0.25 μg of p53) in the presence or absence of 0.5 μg of pCMV-E2F1. Values were normalized to protein levels using the Bio-Rad Dc assay (Bio-Rad) and to the activity of equal micrograms of co-transfected β-galactosidase (Promega). Transfections were performed in H1299 cells (*A*) and in mouse embryo fibroblasts from the p21 knock-out mouse (p21 −/−, *B*). The results depicted are the average of three independent experiments; *error bars* mark S.D.

inducing 4-fold repression of the SpVI construct, the Δ61–75 mutant of p53 was unable to repress this promoter construct (Fig. 8*B*). At higher concentrations of p53 (0.5 μg), however, this Sin3-binding mutant was able to repress the *survivin* promoter, albeit at reduced levels compared with wt p53 (data not shown).

In similar studies we sought to test the significance of the 3-nucleotide spacer in the p53-binding site for repression of Survivin. Specifically, we sought to test the hypothesis that deletion of the 3-nucleotide spacer could convert this element to a p53-transactivated site. Deletion of this spacer, in the background of the p53-repressible Sp-min construct (SpVII), rendered this construct potently transactivated by p53 (Fig. 8*C*). Notably, the Δ61–75 construct of p53, which fails to interact with Sin3, is equally capable of transactivating this construct. The Δ61–75 mutant also transactivated the $p21^{waf1}$ promoter to levels indistinguishable from wt p53 (26 and data not shown). Therefore, deletion of the 3-nucleotide spacer region in the p53-binding site of the *survivin* promoter is sufficient to render this site equivalent to a transactivated element.

Overexpression of Survivin Can Inhibit Apoptosis in Cells with wt p53—To test the contribution of p53-mediated repression of Survivin to apoptosis, it became logical to test the ability of overexpressed Survivin to inhibit apoptosis induced by p53. Toward this end, we generated stable transfectants of CaCl melanoma cells, which are sensitive to p53-dependent apoptosis, with Survivin constructs driven by the cytomegalovirus immediate-early promoter. Because we and others (43) noted that overexpression of wild type Survivin was associated with some toxicity in cells, we used for these studies a Survivin construct that was mutated to mimic constitutive phosphorylation at the threonine residue at amino acid 34. The available data indicate that the threonine 34-phosphorylated form of Survivin is the anti-apoptotic form of this protein (43). Stable transfection of the T34E variant of Survivin yielded several subclones of CaCl cells with greatly overexpressed Survivin protein (20–30-fold overexpression, see Fig. 9). CaCl-Survivin clones, as well as vector-transfected control, were treated with increasing doses of ultraviolet light to induce apoptosis, and the extent of apoptosis after 24 h was monitored by immunopositivity for the caspase-cleaved p85 fragment of poly(ADP) ribose polymerase (PARP). As depicted in Fig. 9, Western analysis of CMV- and Survivin-transfected cells treated with increasing doses of ultraviolet radiation led to significant p53 induction in both cell lines (Fig. 9). However, only in CMV-transfected cells did this p53 induction lead to dose-dependent increases of p85 PARP, a marker for caspase activation and apoptosis. Similar results were obtained in another independently generated Survivin-transfected clone (CaCl-T34E-Survivin clone 1, data not shown). We also noted reduced annexin V staining in UV-treated cells that overexpress Survivin, relative to vector-transfected control (data not show). These data support the hypothesis that overexpression of Survivin can inhibit UV-induced apoptosis and that repression of Survivin by p53 would be predicted to either directly induce, or sensitize cells to, apoptosis.

DISCUSSION

Although originally identified as an IAP (1) and shown to inhibit caspase activity *in vitro* (2), that Survivin is a *bona fide* caspase inhibitor has been the subject of considerable debate. Nonetheless, several groups (4–6) have shown that antisense down-regulation of Survivin is sufficient to induce apoptosis in human tumor cell lines. Interestingly, we found that expression of wt Survivin in human tumor cells was associated with considerable toxicity and that only by mutating this protein at amino acid 34, to mimic phosphorylation at this site (as indicated in Ref. 43), were we able to generate stably transfected cell lines that overexpress this protein. Along these lines it has been proposed that the non-phosphorylated form of Survivin protein may in fact function as a dominant negative protein *in vivo* (43), and this may explain the toxicity evident in our studies. We were able to generate two independent subclones of tumor cells that express 20–30-fold increased levels of the T34E Survivin protein. Notably, both of these cell lines demonstrated resistance to apoptosis induced by ultraviolet light, as determined by reduced cleavage of the protein PARP, a marker for apoptosis. Therefore, although the mechanism

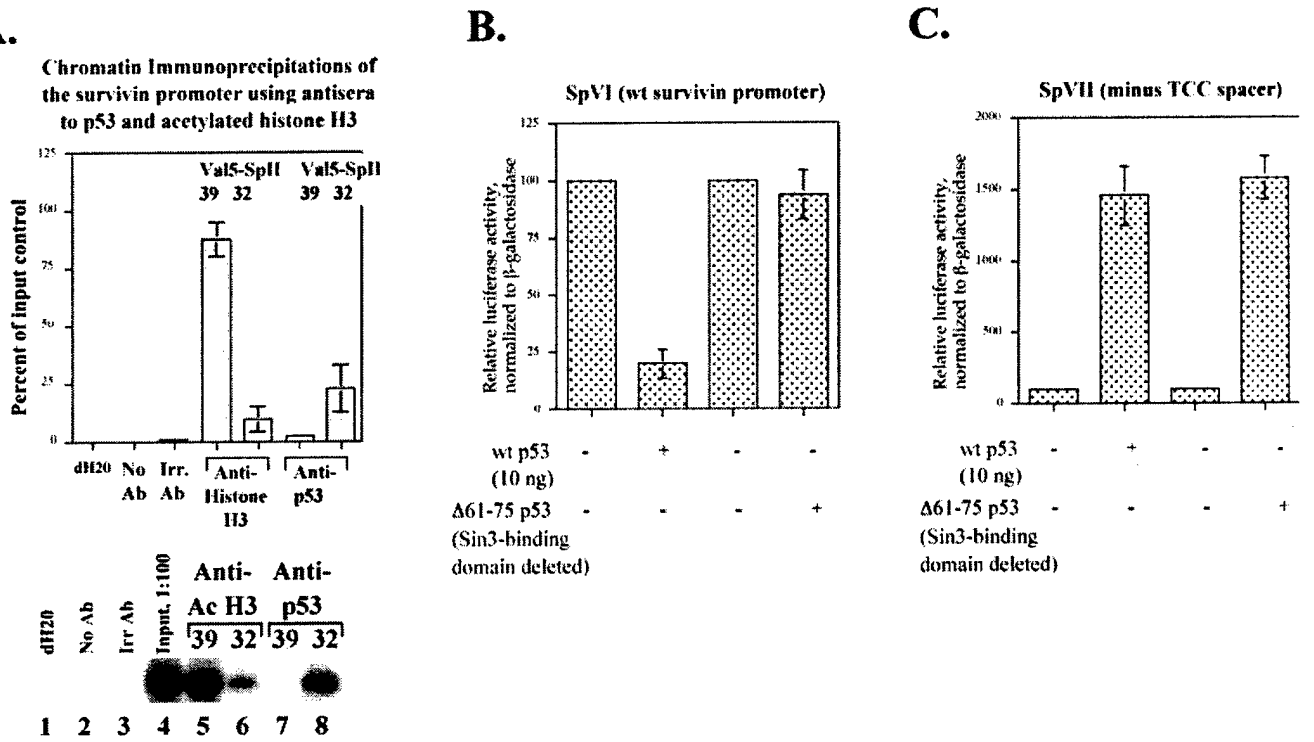


FIG. 8. **p53 binds to the *survivin* promoter *in vivo*; removal of the 3-nucleotide spacer in the *survivin* promoter renders this site transcriptionally induced by p53.** *A*, chromatin immunoprecipitations of the *survivin* promoter in Val5-SpII cells, which are stably transfected with the full-length *survivin* promoter driving the expression of the luciferase gene. Values for the controls (*dH20*, no antibody, irrelevant antibody (419 monoclonal antibody), and 1:100 dilution of input chromatin) are for Val5-SpII cells at 32 °C; all other values are for the temperatures listed. Antisera specific for acetylated histone H3 (Upstate Biotechnology, Inc.) and p53 polyclonal antisera (Santa Cruz Biotechnology) were utilized for these experiments. The data depicted to the *right* are from a single experiment following Southern transfer and hybridization to a probe specific for the *survivin* promoter. The quantitated data are from three independent experiments; mean ± S.E. *error bars* are shown. *B*, transient transfection of the *survivin* promoter construct SpVI and 10 ng of either wt p53 or the p53 mutant that lacks the Sin3-binding domain, amino acids 61–75 (Δ61–75 p53). Whereas wt p53 is capable of repressing the *survivin* promoter, the Sin3-binding domain mutant is unable, indicating that transcriptional repression of *survivin* requires Sin3 and supporting a direct repression of *survivin* by p53. As a control for transfection efficiency and nonspecific repression, equal microgram amounts of SpVI and the SV40-driven β-galactosidase gene were transfected and assayed. The results depicted are the levels of luciferase activity normalized to β-galactosidase and are the averages of three independent experiments where the activity of SpVI in the absence of p53 was set to 100%. *Error bars* mark S.D. in the three experiments. *C*, the *survivin* p53-binding site functions as a transactivated element when the 3-nucleotide spacer element is deleted. The *survivin* construct SpVII was generated by polymerase chain reaction as described under "Experimental Procedures" to recreate the p53-binding element of the *survivin* promoter, minus the TCC spacer. This construct was transfected into p53-null H1299 cells in the absence or presence of 10 ng of wt p53 or the Δ61–75 mutant of p53, which fails to repress transcription from the normal *survivin* promoter. This artificial element is transactivated over 100-fold by either wild type p53 or the Δ61–75 mutant. The averaged data from three independent experiments are depicted, and *error bars* mark S.D. from the three experiments.

whereby Survivin inhibits apoptosis is subject to some debate, this protein clearly functions to inhibit apoptosis induction in this cell line system. Further analysis of these Survivin-overexpressing cells should aid in the clarification of the mechanism whereby this protein inhibits apoptosis.

In this study we have identified the *survivin* gene as a member of a growing class of genes with a role in the G_2/M transition of the cell cycle that are also negatively regulated by p53; these genes include *cdc2*, *cdc25c*, and *cyclin B1* (28, 29, 41). The promoters of these genes, like *survivin*, each contain a bipartite element near the start site of transcription, termed a CDE/CHR (cell cycle-dependent element/cyclin homology region). This element interacts with an as yet uncloned binding protein termed CDF-1 in the G_1 phase of the cell cycle; this binding is believed to lead to transcriptional repression of these genes in G_1 and enhanced expression in G_2/M (44, 45). Whereas this element is clearly important for the cell cycle-regulated expression of these genes, our data indicate that the CDE/CHR element is not required for the repression of *survivin* promoter constructs by p53 in transient assays. Additionally, we have found that G_1 arrest alone, induced by the p53-homologue p73, is not sufficient to cause repression of *survivin*.

Two mechanisms for the p53-mediated repression of genes like *cdc2*, *cdc25c*, and *cyclin B1* have been proposed. Repression of these genes by p53 has been proposed to involve inhibition by p53 of the NF-Y transcription factor, which binds to CCAAT boxes in these promoters and normally transactivates these genes (39, 46, 47). As the *survivin* promoter does not contain an obvious CCAAT box-binding site for NF-Y, it is unlikely that this mechanism plays a role in p53-dependent repression of *survivin*. It has also been proposed that p53-dependent repression of *cdc2* by p53 relies on up-regulation of $p21^{waf1}$. This leads to hypophosphorylation of pRB family proteins and transcriptional repression via E2F family members (29). Our data indicate that p53 can repress *survivin* in p21-null cells, which would argue against such a mechanism; however, we have consistently noted that the repression of *survivin* by p53 shows decreased magnitude in cells lacking p21 or pRB. Therefore, it is possible that p53 represses *survivin* in part by transactivation of $p21^{waf1}$ and subsequent conversion of E2F complexes to E2F-RB repressor complexes. Our data indicate, however, that this may be one of two overlapping, redundant mechanisms for the repression of *survivin*.

In this study we show that p53 binds *in vivo* to the *survivin* promoter to a consensus p53-binding site, raising the possibility that p53 represses *survivin* by interfering with E2F-mediated transactivation. We have found that binding of p53 to this region is accompanied by a decreased association of this pro-

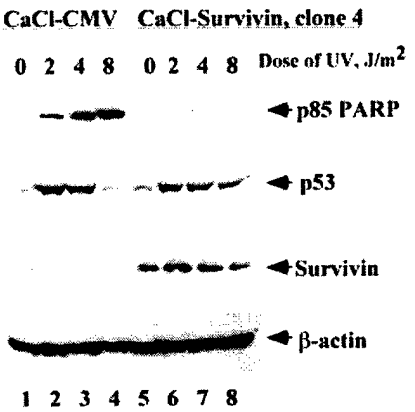


FIG. 9. **Overexpression of Survivin (T34E) inhibits PARP cleavage following apoptosis induced by ultraviolet radiation.** Western analysis of clones of human CaCl melanoma cells stably transfected with vector alone (*CaCl-CMV*) or a CMV-driven Survivin T34E construct (*CaCl-Survivin clone 4*; amino acid 34 of Survivin has been mutated to glutamic acid to mimic phosphorylation of this residue, see "Experimental Procedures"). Clones were subjected to ultraviolet radiation at the does indicated and harvested after 24 h, at which time they were subject to Western analysis for antibodies specific to the caspase-cleaved form of PARP (p85 PARP, Promega), p53, Survivin, and β-actin. Although p53 induction is comparable in both cell lines, only CaCl-CMV cells, and not cells overexpressing Survivin, show an increase in the presence of p85 PARP, which is a marker for apoptosis. Representative data from a single experiment are shown and are consistent with results from two independently derived subclones of CaCl-Survivin cells (clone 1 and clone 4).

moter with acetylated histones; this would be consistent with the action of the p53-Sin3-HDAC complex. We propose a model whereby direct binding of p53 to the *survivin* promoter confers repression by p53, via the p53-Sin3-HDAC complex. Interestingly, we have found that, when placed alone upstream of a minimal promoter, the p53-binding site of the *survivin* promoter is not sufficient to confer repression to a heterologous gene. Rather, the overlapping E2F-site is required along with the p53-binding site.[2] Therefore, we favor the hypothesis that the p53-Sin3-HDAC complex binds to the *survivin* promoter and modifies the chromatin conformation such that E2F-binding and/or transactivation is impaired or abrogated. Such a mechanism of repression by p53 has been seen for other promoters, such as the α-fetoprotein promoter, on which p53 binds and competes for binding with the HNF-3 transcription factor (48).

It is formally possible that p53 and E2F proteins occupy the *survivin* promoter at the same time and together create a transcriptional repressor. Indeed, p53 and E2F-1 have been reported to interact, and association with p53 has been reported to inhibit transactivation by E2F-1 (49). Overall, the combined data favor both direct (p53 binding, histone deacetylation) and indirect (induction of $p21^{waf1}$) mechanisms for the repression of *survivin* by p53. That p53 uses both direct and indirect mechanisms of repression of the *survivin* promoter may simply reflect the existence of back-up mechanisms to ensure efficient and timely repression. Alternatively it is possible that each mechanism may be specific to particular stresses, phases of the cell cycle, or cell types.

We have identified a p53-binding site in the *survivin* promoter that conforms to the consensus found for the first p53-binding sites identified (50). This consensus site was defined as two copies of the sequence 5′-RRR-C(A/T)(T/A)G-YYY-3′ (where Pu is ? and Pyr is ?), separated by 0–13 base pairs; functional p53-binding elements can contain up to four mismatches in this consensus, but the C and G residues are invariant (50). The human *survivin* promoter contains a spacer of 3 nucleotides; this site is conserved in the murine promoter, which has two overlapping p53-binding sites, one with a 4-nucleotide spacer and one with a 2-nucleotide spacer. Whereas the consensus p53-binding site identified by El Deiry and coworkers (50) contained a spacer of 0–13 nucleotides, the functionally defined p53 sites contained within p53-induced genes typically contain spacer elements of less than 2 nucleotides. In fact, increasing the spacer from 1 to 4 nucleotides has been shown to eliminate the ability of p53 to transactivate from this site; interestingly, increasing the spacer to 10 nucleotides restores transactivation (40). We propose that increasing the spacer changes the orientation of the p53 dimers to each other and that transactivation can occur only if the dimers are on the same face of the DNA helix. Otherwise, this site functions passively as a p53-binding site and is inactive for transactivation; instead p53 bound to this site may interfere with the binding or activity of other transcription factors, such as E2F-1. That the DNA-binding domain of p53 is able to accommodate such spacing changes in the DNA-binding site is supported by the fact that a flexible linker region connects the DNA binding domain to the oligomerization domain (51). Similar subtle changes in the p53 consensus element have been shown to influence the p53 transcriptional response (52, 53). Interestingly, two p53-binding sites, both with 3-nucleotide spacers, are present in the promoter of the *p202* gene, which has recently been identified as a p53-repressed gene (54). The contribution of this type of binding site to the repression of the *p202*, and other p53-repressed, promoters remains the subject of future study.

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[2] W. H. Hoffman and M. Murphy, unpublished observations.

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Activation of Cancer-Specific Gene Expression by the Survivin Promoter

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Background: **Survivin, a member of the IAP (inhibitor of apoptosis) gene family, appears to be overexpressed in common cancers but not in corresponding normal adult tissues. To investigate whether the survivin promoter controls cancer cell–specific gene expression, we determined whether the survivin gene promoter could regulate reporter gene expression in cancer cell lines and xenografts.** ***Methods:*** **Survivin protein levels were determined in human and murine cancer cell lines and in normal tissues of adult C57BL/6 mice by Western blot analysis. A reporter construct in which a portion of the survivin gene promoter was used to drive transcription of a human secreted alkaline phosphatase (SEAP) gene was transiently transfected into cancer cells, and promoter activity was extrapolated from SEAP activity. A2780 human ovarian cancer cells were transfected with this construct, and stable transfectants were injected into the intrabursal ovarian space of immunodeficient mice. Tumor growth was monitored, and plasma SEAP levels were used as a measure of survivin promoter activity *in vivo*.** ***Results:*** **Survivin protein was detected in all cancer cell lines examined but not in most normal adult mouse tissues. After transfection, the survivin promoter was more active in all cancer cell lines than in normal ovarian surface epithelial cells or mouse 3T3 cells. After 0.8×10^6 stable transfectant cells were injected into the intrabursal cavity of mouse ovaries, plasma SEAP activity was detected within 24 hours, and the activity increased with time and tumor growth.** ***Conclusion:*** **Transfection experiments indicate that survivin protein expression in cancer tissue appears to be regulated, at least in part, transcriptionally. Thus, the survivin promoter may be useful in controlling gene expression in cancer cells. [J Natl Cancer Inst 2002;94:522–8]**

Members of the inhibitors of apoptosis (IAP) gene family may play important roles in the survival of cancer cells and the progression of malignancies. The first IAP isolated was the product of a baculovirus gene. Other members of this gene family, including survivin *(1)*, have subsequently been identified in many species, including humans *(2)*. Genes for members of the IAP family are generally characterized by one or more copies of the so-called baculovirus IAP repeat and by a ring finger domain at their carboxyl terminus *(3)*. The survivin gene, located on the long arm of human chromosome 17 at band 25, contains a single baculovirus IAP repeat but no ring finger motif. Because survivin inhibits apoptosis in mammalian cells, the ring finger may not be required for all IAP functions, at least in mammals *(3)*.

Although survivin is not expressed in normal adult human tissues, it is expressed in various human cancers *(4,5)*. Survivin expression may be activated transcriptionally *(6)*; consequently, the survivin promoter might be a cancer-specific promoter with utility in gene therapy or oncolytic viral replication. Such a tumor-selective promoter may also be useful in tumor-prone transgenic mice by activating the expression of a marker gene at the initiation of oncogenesis. In this study, we used transfection experiments to examine whether 1092 base pairs of the 5′ upstream regulatory sequence of the human survivin gene could control the expression of a reporter gene in cancer cell lines derived from tumors of the uterine cervix, breast, ovary, lung, and colon. We also evaluated the activity of the survivin promoter in a cancer cell line *in vivo* as a xenograft.

Materials and Methods

Cell Lines and Cell Culture

The following human cancer cell lines (and their tissue of origin) were used in this study: A2780, OVCAR3, OVCAR5, OVCAR8, OVCAR10, SKOV3, PEO1, and UPN251 (ovary); HT29 (colon); MCF7 (breast); H1299 (lung); and HeLa (uterus). ROSE-TAg is a tumorigenic cell line derived from Fisher 344 rat ovarian surface epithelial (ROSE) cells transformed with simian virus 40 (SV40) large T antigen (TAg) *in vitro*. NuTu19 and NuTu26 are spontaneously transformed cell lines derived from Fisher 344 ROSE cells *(7)*. IG10 and IF5 are spontaneously transformed mouse ovarian surface epithelial (MOSE) cell lines *(8)*. Mc6 is a mammary cancer cell line derived from a mammary tumor of a mammary tumor-prone C3 (1)/TAg transgenic mouse line, and Pr14 is a prostate cancer cell line derived from a prostate tumor of a prostate cancer-prone line of C3 (1)/TAg transgenic mice *(9,10)*. Normal MOSE cells were isolated from the ovaries of C57BL/6 adult mice *(8)* and used for up to three passages. Normal ROSE cells were isolated from the ovaries of Fisher 344 rats *(7)* and used for up to five passages. Normal human ovarian surface epithelial (HOSE) cells were derived from normal human ovaries as previously described *(11)*. NIH 3T3 is an immortalized, nontumorigenic mouse fibroblast cell line. All cell lines were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2/95% air. HOSE, A2780, OVCAR3, OVCAR5, OVCAR8, OVCAR10, SKOV3, PEO1, UPN251, ROSE-TAg, NuTu19, NuTu26, MCF7, and normal ROSE cells were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). IG10, IF5, Mc6, Pr14, and MOSE cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL) plus 5% FBS. NIH 3T3 cells were cultured in

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See "Notes" following "References."

DMEM plus 10% calf serum, and H1299 and HeLa cells were cultured in DMEM plus 10% FBS. All media were supplemented with streptomycin (100 μg/mL), penicillin (100 U/mL), glutamine (0.3 mg/mL), and pork insulin (0.25 U/mL or 1× ITS [insulin, transferrin, and selenium]; GIBCO BRL, Rockville, MD).

Western Blot Analysis

At about 70% confluence, cells were harvested with trypsin/EDTA, and PBS-washed cell pellets were stored at −70 °C until use. Whole-cell protein was extracted from the cell pellets with RIPA buffer (50 m*M* Tris–HCl at pH 7.4, 150 m*M* NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate). Protein was also extracted from normal tissues (brain, thymus, heart, lung, liver, stomach, small intestine, bladder, kidney, ovary, oviduct, uterus, spleen, pancreas, and skeletal muscle) of female C57BL/6 mice (8 weeks, 12 months, or 16 months of age). Tissues were homogenized in a Mini Bead Beater (BioSpec Products, Inc., Bartlesville, OK) and the T-MER tissue protein extraction reagent (Pierce, Rockford, IL). For survivin detection, 30 μg of total protein extract was resolved on sodium dodecyl sulfate–15% polyacrylamide gels and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). The blots were hybridized with an anti-survivin polyclonal antibody (diluted 1 : 2000; Novus Biological, Littleton, CO), followed by incubation with a peroxidase-conjugated goat anti-rabbit second antibody (diluted 1 : 5000; Amersham). The peroxidase activity was detected by the ECL method (NEN, Boston, MA).

Vector Construction

A 1092-base-pair fragment of the human survivin gene promoter (nucleotides 1821–2912, GenBank accession number U75285) was excised from plasmid SpI with restriction enzymes *Kpn*I and *Xho*I *(12)*. The secreted alkaline phosphatase (SEAP) expression vector under control of the survivin promoter (pSRVN-SEAP) was constructed by subcloning the *Kpn*I-*Xho*I fragment into the multiple cloning site of the SEAP expression vector pSEAP-Basic (Clontech, Palo Alto, CA). To generate stable transfectants, the pSRVN-SEAP-NEO plasmid was constructed by subcloning the SRVN-SEAP sequence (a *Kpn*I-*Xba*I fragment) from pSRVN-SEAP into the PC3 vector *(13)*, a modified pcDNA3 vector (Invitrogen, San Diego, CA) without the cytomegalovirus promoter.

Transient Transfection

The pSRVN-SEAP plasmid was transiently transfected into cell lines by use of the TransIT-LT1 transfection reagent (Pan-Vera, Madison, WI). Briefly, 3×10^5 cells were placed into each well of a six-well plate in 2 mL of complete medium. After incubation overnight, cells were 40%–50% confluent, and a mixture of 2 μg of pSRVN-SEAP plasmid, 0.2 μg of pGL3 control plasmid, 6 μL of LT1 transfection reagent, and 100 μL of serum-free medium was added to each well. The pGL3 control plasmid (Promega, Madison, WI), which is a luciferase expression vector driven by the SV40 promoter, was used to assess transfection efficiency and, hence, normalize each transfection. Two other plasmids, pSEAP-Basic (a promoterless SEAP construct) and pSV40-SEAP (a SEAP expression vector with the SV40 promoter) (Clontech), were also used for each cell line as negative and positive controls, respectively. Medium (100 μL) was removed 48 hours after transfection and used to determine SEAP activity after normalization of the transfection efficiency. Briefly, the adherent cells were washed once with PBS, exposed to 1 mL of lysis buffer (Promega), and scraped from dishes with a cell scraper. After centrifugation of the cell lysates at 15 700 relative centrifugal force (rcf) for 1 minute, the supernatants were removed and stored at −70 °C until luciferase activity was assayed. Luciferase activity was determined by mixing 5 μL of supernatant with 100 μL of luciferase assay reagent (Promega) and determining the relative luminescence with a luminometer (Analytical Luminescence Laboratory, San Diego, CA). This procedure allowed us to adjust the amount of conditioned medium used to allow for differences in transfection efficiency.

Stable Transfection

The pSRVN-SEAP-NEO plasmid was linearized with restriction enzyme *Pvu*I and purified by phenol–chloroform extraction and ethanol precipitation. Before electroporation, subconfluent A2780 cells were trypsinized, washed twice with PBS, and resuspended at 10×10^6 cells in 0.7 mL of PBS. The cell suspension was transferred into a Gene Pulser cuvette (Bio-Rad Laboratories, Hercules, CA), and 5 μg of linearized pSRVN-SEAP-NEO or control vector PC3 was added. After 10 minutes on ice, the cells were subjected to electroporation by using the Gene Pulser II System (Bio-Rad Laboratories) at 250 V/cm and 975 μF and then plated in three 10-mm Petri dishes with complete medium. One day later, medium was changed to complete growth medium supplemented with G418 at 500 μg/mL. After 2 weeks, the G418-resistant clones were isolated with cloning cylinders. SEAP activity in the conditioned medium from individual clones was determined when the cells were nearly confluent.

Animal Study

Female CB17/ICR SCID (severe combined immunodeficient) mice, approximately 8 weeks of age and weighing approximately 20 g, were used to establish orthotopic ovarian tumors. All these mice were bred in the Laboratory Animal Facility at Fox Chase Cancer Center, were maintained in specific pathogen-free conditions, and received commercial food and water *ad libitum*. Institution guidelines were followed in handling the animals. To establish the orthotopic tumors, cultured A2780 transfectants (two SRVN-SEAP-NEO clones, $A2780^{SSN1}$ and $A2780^{SSN2}$, and one vector control clone, $A2780^{PC3}$) were harvested with 0.05% trypsin-EDTA (GIBCO BRL), washed in PBS, and resuspended in RPMI-1640 complete medium at 40×10^6 cells per milliliter. Before intrabursal implantation of tumor cells, eight SCID mice were anesthetized with a 15 : 3 : 5 : 152 mixture of ketamine-HCl (100 mg/mL), acepromazine malleate (10 mg/mL), xylazine hydrochloride (20 mg/mL) (Fort Dodge Animal Health, Fort Dodge, IA), and 0.9% normal saline, injected intraperitoneally at 10 μL/g of body weight. The skin was disinfected with Wescodyne and 70% ethanol. A small incision was made on one side of the back to locate the ovary. The oviduct was held with small forceps, and a 26-gauge needle connected to a syringe was inserted into the oviduct and was passed through the infundibulum until the needle tip reached the space between the bursa and the ovary. Approximately 20 μL of the cell suspension (about 0.8×10^6 cells) was injected into the intrabursal space. The needle was slowly removed, the ovary

was replaced in the abdominal cavity, and the body wall was closed with sutures. One ovary of each animal was injected.

Plasma for SEAP analysis was obtained by orbital puncture with heparinized glass tubes (Fisher Scientific, Pittsburgh, PA) on days 0, 1, 3, 6, and 9 after cell implantation. About 20 μL of plasma was obtained after the blood was centrifuged at 4500 rcf for 7 minutes *(14)*. Animals were sacrificed 14 days after implantation; ovaries were removed, embedded in paraffin, and sectioned for histopathologic analysis.

SEAP Assay

SEAP activity in culture medium or plasma was determined by a chemiluminescence or fluorescence method using Great Escape SEAP kits from Clontech *(15)*. In brief, 5-μL samples were mixed with 45 μL of dilution buffer and incubated in a oven at 70 °C for 45 minutes. Sixty microliters of assay buffer containing L-homoarginine was then added. After a 5-minute incubation at room temperature, the samples were exposed to 60 μL of chemiluminescent substrate CSPD (disodium 3-[4-methoxyspiro{1.2-dioxetane-3,2′-(5′-chloro)tricyclo(3.3.1.1)-decan}4-yl]phenyl phosphate) (1.25 mM) or 3 μL of fluorescent substrate 4-methylumbelliferyl phosphate (Clontech). Chemiluminescence was measured with a luminometer (Analytical Luminescence System) after a 10-minute incubation at room temperature.

After a 60-minute incubation in the dark, fluorescence was measured with a CytoFluor II fluorometer (Bio-Rad Laboratories) with excitation and emission wavelengths of 360 nm and 449 nm, respectively. SEAP activity was determined from a standard curve.

To determine whether exogenous SEAP could be separated from endogenous placental alkaline phosphatase of pregnant animals, plasma from two C57BL/6 pregnant mice at embryonic day 12, one normal control mouse, and one CB17/ICR SCID mouse carrying an A2780^{SEAP13} cell implant *(14)*, was isolated. Five microliters of plasma was mixed with 45 μL of dilution buffer, and the mixture was heated to 70 °C for 0, 20, 40, or 60 minutes. Alkaline phosphatase activity was determined as described above.

Results

Survivin Protein Levels in Cancer Cell Lines, Normal Cells, and Normal Tissues

To determine how frequently the increased expression of survivin is detected in cancer cell lines compared with normal tissues and cells, protein extracts were prepared from cells and tissues derived from different species and subjected to western blot analysis. As shown in Fig. 1, A, survivin protein was detected as an intense band at 16.5 kDa in all the human ovarian cancer cell lines, including A2780, OVCAR3, OVCAR5,

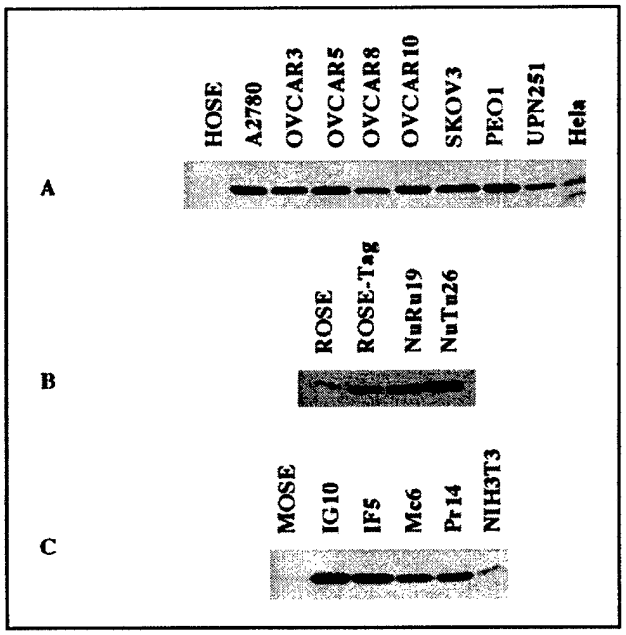

Fig. 1. Western blot analysis of survivin protein in cell lines from various species. **A)** Survivin protein level in human ovarian cancer cell lines (A2780, OVCAR3, OVCAR5, OVCAR8, OVCAR10, SKOV3, PEO1, and UPN241) was compared with that in normal human ovarian surface epithelial (HOSE) cells. The survivin-expressing cell line HeLa from uterine cervix was the positive control. **B)** Survivin protein level in transformed rat ovarian surface epithelial cell lines (ROSE-Tag, NuRu19, and NuRu26) was compared with that in early-passage rat ovarian surface epithelial (ROSE) cell lines. **C)** Survivin protein level in transformed mouse ovarian surface epithelial cell lines (IG10 and IF5), mammary (Mc6) and prostate (Pr14) tumor cell lines from transgenic mice, and a mouse fibroblast cell line (NIH 3T3) was compared with that in early-passage mouse ovarian surface epithelial (MOSE) cells.

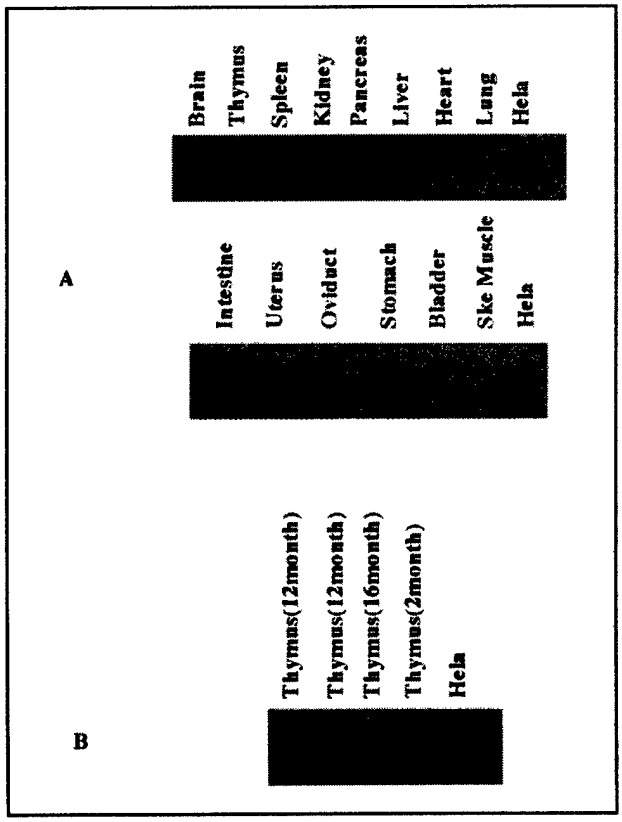

Fig. 2. Western blot analysis of survivin protein in tissues from C57BL/6 mice as indicated. The uterine cervix cancer cell line HeLa was used as the positive control. **A)** Survivin protein level in tissues of 8-week-old C57BL/6 mice. **B)** Survivin protein level in thymus from mice aged 2, 12, and 16 months. Ske = skeletal.

OVCAR8, OVCAR10, SKOV3, PEO1, and UPN251, but not in the normal HOSE cells. An intense 16.5-kDa survivin band was also detected in transformed rat ovarian surface epithelial cell lines (ROSE-TAg, NuTu19, and NuTu26), but only a faint band was detected in early-passage normal ROSE cells (Fig. 1, B). Consistent with our findings in normal and transformed ROSE cells, an intense survivin band was detected in the transformed MOSE cell lines (IG10 and IF5), but only a faint band was detected in the normal MOSE cells (Fig. 1, C). An intense survivin band was also detected in tumor cell lines from transgenic mice prone to develop mammary tumors (Mc6) *(9)* or prostate tumors (Pr14) *(10)*. A faint survivin band was detected in nontumorigenic mouse fibroblast cell line NIH 3T3 (Fig. 1, C).

Survivin Protein Levels in Normal Adult Mouse Tissues

Levels of survivin protein in normal tissues from 8-week-old female C57BL/6 mice were determined by western blot analysis. No survivin protein was detected in brain, heart, lung, liver, stomach, intestine, bladder, kidney, ovary, oviduct, uterus, pancreas, or skeletal muscle. Survivin was detected in the thymus and, to a lesser extent, in the spleen (Fig. 2, A). Because age-associated thymic atrophy could result in a decrease in survivin protein in the thymus, we evaluated survivin expression in 12-month-old and 16-month-old C57BL/6 mice and detected a marked reduction of survivin protein in mature, as opposed to young adult, mice (Fig. 2, B).

In Vitro Survivin Promoter Activity

We constructed the pSRVN-SEAP plasmid to determine whether the survivin promoter functioned in cancer cells. Promoter activity was determined from the SEAP activity in conditioned medium from transiently transfected cells. In A2780 cells transfected with the promoterless pSEAP-Basic plasmid, SEAP expression was almost baseline (Table 1). In several other cancer cell lines, SEAP expression was also almost baseline, but in others, the promoterless plasmid had some activity. In all cancer cell lines transfected with a plasmid containing the survivin promoter (i.e., pSRVN-SEAP), SEAP expression was five-fold to about 400-fold higher than that observed with the promoterless pSEAP-Basic plasmid (Table 1; Fig. 3, A). However, early-passage normal ROSE and MOSE cells similarly transfected showed less SEAP expression when transfected with pSRVN-SEAP than with pSEAP-Basic (Table 1). To determine the relative promoter activity of the survivin promoter compared with the relatively strong SV40 viral promoter, we transfected the various cell lines with the SV40 promoter-driven SEAP expression plasmid pSV40-SEAP and measured SEAP expression. The survivin promoter was more active in the cancer cell lines, and the SV40 promoter was more active in the nontransformed cell lines (i.e., NIH 3T3, ROSE, and MOSE cells) (Table 1; Fig. 3).

In Vivo Survivin Promoter Activity

To determine whether the survivin promoter could induce enough SEAP activity to monitor tumor growth *in vivo*, we created stable A2780 transfectants harboring stably integrated SRVN-SEAP-NEO. Two clones ($A2780^{SSN1}$ and $A2780^{SSN2}$) were selected because of their relatively high SEAP production (Fig. 4, A). These two SRVN-SEAP-NEO clones and one vector control clone ($A2780^{PC3}$) were used to generate orthotopic ovarian tumors by injection into the intrabursal space of mouse ovaries to mimic early ovarian cancer. After tumor cell implantation, plasma was collected at designated intervals to measure SEAP activity. SEAP activity was detected as early as 24 hours in animals implanted with 0.8×10^6 cells from either of the two SRVN-SEAP-NEO clones and increased with time and tumor growth. In contrast, SEAP activity was not detected in the animal injected with the vector control clone (Fig. 4, B and C). Paraffin sections prepared from ovaries removed on day 14 had small tumors in the intrabursal cavity in all mice injected with a pSRVN-SEAP-NEO clone ($A2780^{SSN}$) or the vector control clone ($A2780^{PC3}$) (Fig. 4, D). Contralateral ovaries were normal.

Sensitivity of Endogenous and Exogenous Alkaline Phosphatase to Heat Treatment

To determine whether endogenous alkaline phosphatase activity could be separated from transgenic SEAP activity, we used heat to inactivate the endogenous activity. Plasma from normal control mice, pregnant mice, and a mouse carrying $A2780^{SEAP13}$ cells *(14)* was treated with heat for 0, 20, 40, or 60 minutes, and

Table 1. Activity of survivin promoter relative to a promoterless or simian virus 40 promoter-driven secreted alkaline phosphatase plasmid in cell lines with various origins*

Cell type	pSEAP-Basic (1)	pSRVN-SEAP (2)	pSV40-SEAP (3)	Ratio (2)/(1)	Ratio (2)/(3)
A2780	100 ± 20	16 000 ± 900	5400 ± 400	160	3
OVCAR3[s]	5000 ± 600	67 000 ± 12 000	40 000 ± 1100	13	2
SKOV3	80 ± 30	41 000 ± 1800	20 000 ± 1300	513	2
NuTu19	20 ± 1	5500 ± 400	3100 ± 200	275	2
IF5	200 ± 20	3000 ± 900	1400 ± 100	15	2
IG10	200 ± 10	1200 ± 30	600 ± 20	6	2
Mc6	300 ± 40	11 000 ± 800	4300 ± 300	37	3
Pr14	50 ± 0	1000 ± 10	600 ± 30	20	2
MCF7[s]	1500 ± 200	160 000 ± 4500	84 000 ± 5600	107	2
HeLa[s]	43 000 ± 3700	8 800 000 ± 17 000	4 000 000 ± 230 000	204	2
HT29[s]	21 000 ± 400	8 800 000 ± 6300	4 600 000 ± 48 100	409	2
H1299[s]	95 000 ± 1200	8 400 000 ± 250 000	4 200 000 ± 110 000	90	2
NIH 3T3	80 ± 20	600 ± 50	1100 ± 200	8	<1
ROSE[s]	15 000 ± 3700	8100 ± 2700	26 000 ± 2300	<1	<1
MOSE[s]	14 000 ± 2000	110 000 ± 18 000	430 000 ± 47 000	8	<1

*Secreted alkaline phosphatase analyses (indicated with a superscripted "s") were done by the luminescence method and expressed as relative luminescence units. Other analyses were done by the fluorescence method and expressed as relative fluorescence units. ROSE = rat ovarian surface epithelial; MOSE = mouse ovarian surface epithelial; SEAP = selected alkaline phosphatase; SV40 = simian virus 40.

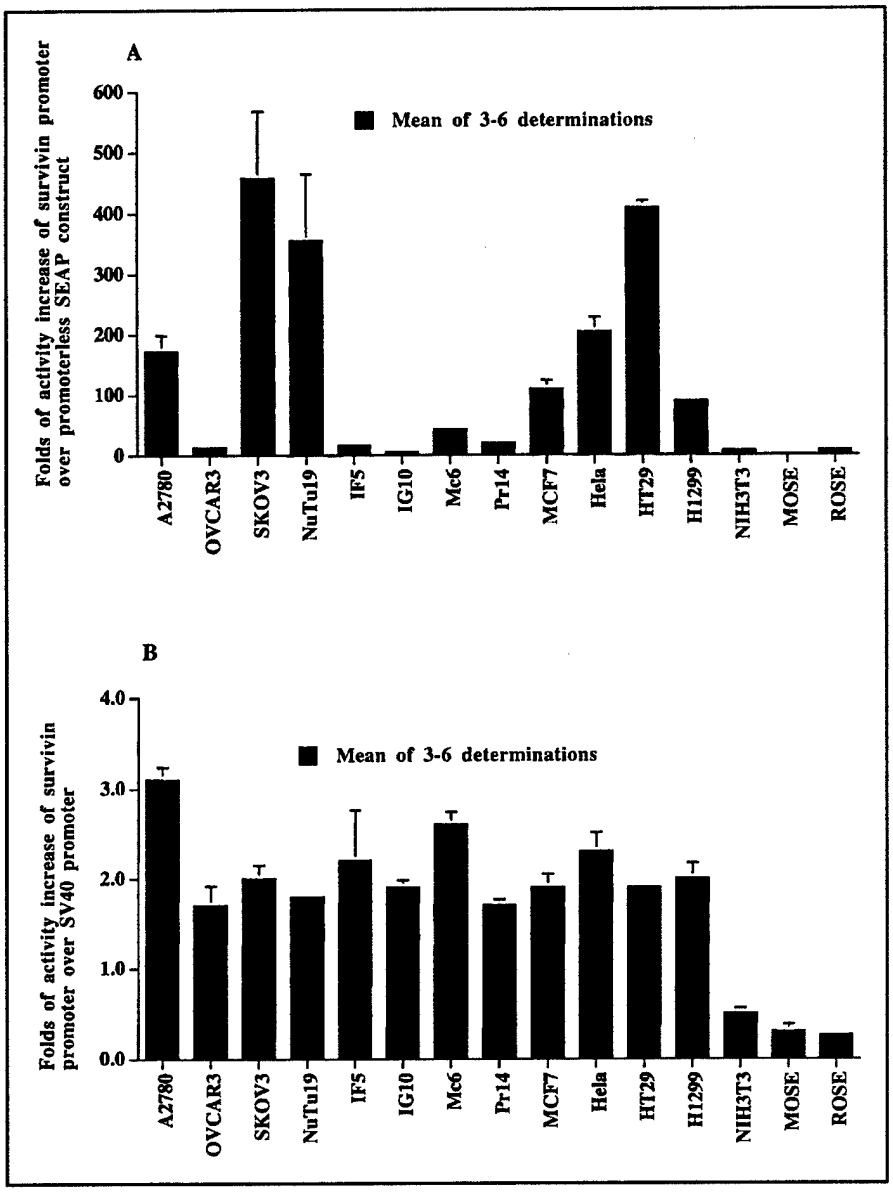

Fig. 3. Activity of the survivin promoter in cell lines as indicated. **A)** Activity of the survivin promoter relative to the activity of the promoterless secreted alkaline phosphatase (SEAP) construct. Activity of the promoterless SEAP construct was defined as 1. **B)** Activity of the survivin promoter relative to the activity of the simian virus 40 (SV40) promoter. Activity of the SV40 promoter was defined as 1. Rose = rat ovarian surface epithelial; MOSE = mouse ovarian surface epithelial. All values are the mean of three to six determinations. **Error bars** are 95% confidence intervals.

alkaline phosphatase activity was determined. As shown in Fig. 5, alkaline phosphatase activity in plasma of normal and pregnant mice decreased quickly after the heat treatment at 70 °C and was still low 40 minutes later. However, plasma alkaline phosphatase activity in the mouse xenografted with $A2780^{SEAP13}$ cells had not decreased after 60 minutes of heat treatment. Therefore, exogenous SEAP activity can be monitored during tumor development and effectively separated from endogenous alkaline phosphatase activity by heat treatment.

Discussion

Regulated induction of apoptosis preserves normal homeostasis and organ morphogenesis. Aberration of this process may contribute to cancer development by prolonging cell viability. Members of the IAP gene family have emerged as unique modulators of apoptosis, possibly by the direct inhibition of terminal effector caspases 3, 7, and 9. Survivin, a new member of the human IAP family, was identified by hybridization screening of human genomic libraries with the complementrary DNA for effector cell protease receptor-1, a factor Xa receptor *(1)*. Unlike other IAP family members, survivin contains a single baculovirus IAP repeat and no carboxyl-terminal ring finger region. Most importantly, at variance with other IAPs such as BCL2, which is present in both normal and transformed cell types, survivin was originally reported to be completely undetectable in normal human adult tissues but expressed during fetal development *(1,16,17)*. Our data on the mouse (this report) and in a published report *(18)*, however, indicate that survivin is present in the thymus and spleen of young adult mice. It seemed unlikely that this difference was related to differences between survivin promoters in mice and humans, because the homology in this region

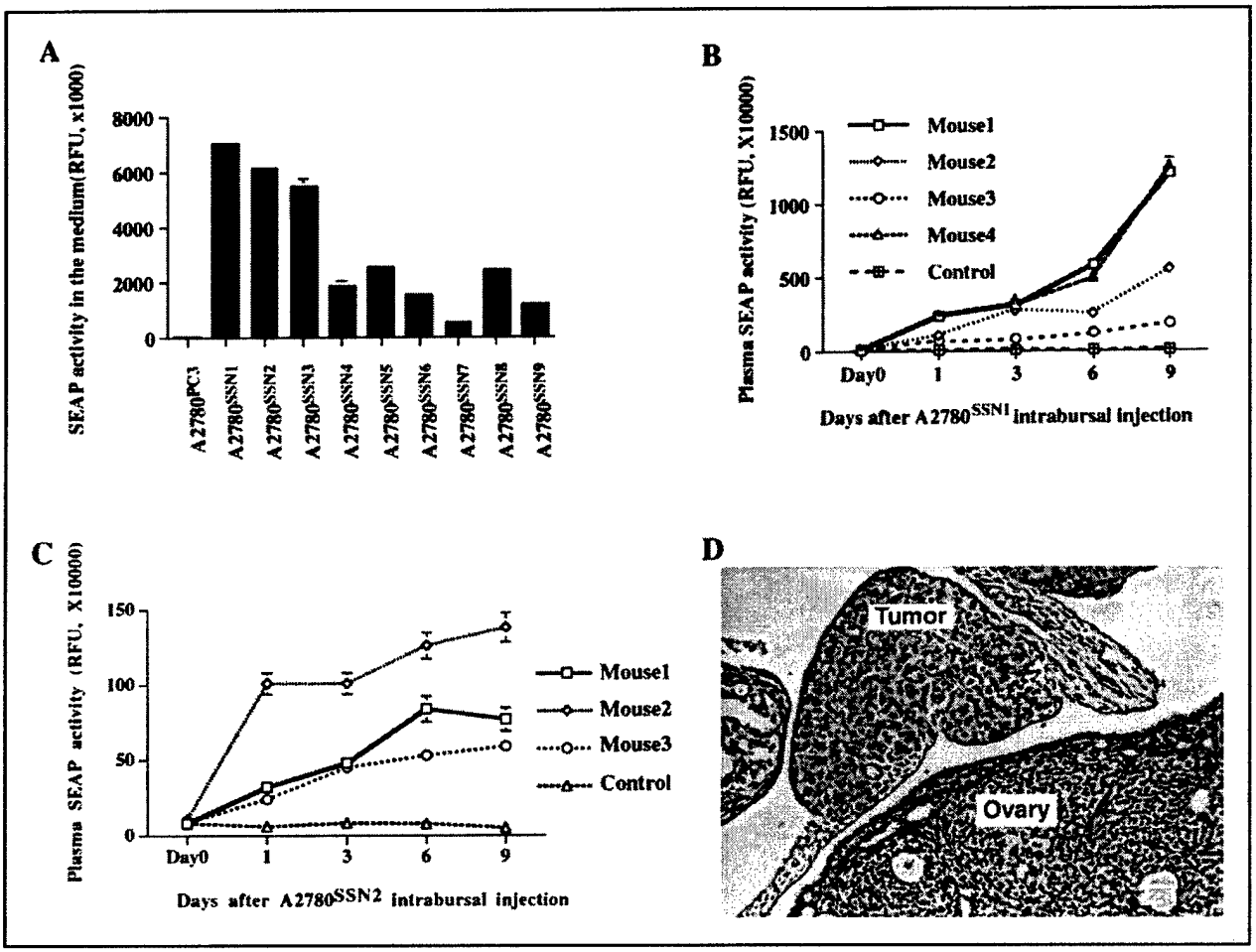

Fig. 4. Activity of the survivin promoter in stable A2780 transfectants. **A)** *In vitro* activity of survivin promoter in nine stable transfectants of A2780 ($A2780^{SSN1-9}$), assessed as secreted alkaline phosphatase (SEAP) activity, compared with that in A2780 transfected with promoterless vector ($A2780^{PC3}$). RFU = relative fluorescence units. **B)** *In vivo* activity of survivin promoter after 0.8×10^6 $A2780^{SSN1}$ cells were injected into the ovarian intrabursal space of severe combined immunodeficient (SCID) mice. The control animal was injected with 0.8×10^6 A2780 cells stably transfected with promoterless vector ($A2780^{PC3}$). **C)** *In vivo* activity of the survivin promoter after another stable transfectant $A2780^{SSN2}$ was injected into the ovarian intrabursal space of SCID mice. **D)** Section of mouse ovary stained with hematoxylin–eosin. The mouse was injected with 0.8×10^6 $A2780^{SSN1}$ cells and killed 14 days after tumor implantation. Data are the mean of three determinations. **Error bars** are 95% confidence intervals.

of the mouse and human genes is high *(6)*. The discrepancy, however, could be related to age. Our initial analysis used young adult mice before the onset of thymic atrophy, as did the earlier report *(18)*. Consequently, we investigated whether an age-related change in survivin expression occurred in the mouse thymus and found that the level of survivin protein was markedly lower in older adult mice than in younger adult mice.

A SAGE (serial analysis of gene expression) analysis found that survivin transcripts were the fourth most frequently overexpressed transcript in common human cancers (e.g., melanoma and cancers of the colon, brain, breast, and lung) relative to levels in normal cells *(5)*, suggesting that survivin was a potential target for cancer therapy. If increased survivin activity is controlled transcriptionally, then the survivin promoter might control transgene expression in a cancer-specific manner. Transcriptional regulation of survivin expression in cancer cells has indeed been reported *(6)*. Using approximately 1 kilobase of the 5′ upstream regulatory region of the survivin gene to drive SEAP expression in ovarian, mammary, colon, lung, and uterine cervical cancer cell lines, we have shown that the survivin promoter can control gene expression regardless of tumor type, mechanism of oncogenesis, and species, and we have confirmed that survivin expression appears to be, at least in part, transcriptionally activated.

In contrast to adult tissues, where survivin expression is largely limited to activation during oncogenesis, in the human fetus, survivin is abundantly expressed in apoptosis-regulated tissues. Similarly, survivin was nearly ubiquitously expressed in embryonic mouse tissues at an early gestational stage (embryonic day 11.5) but was later expressed more selectively *(16)*. Increased survivin expression and survivin promoter activity in cancer cell lines indicate that transcriptional factors needed for survivin transcription reappear or are reactivated during oncogenesis. The approximately 1-kilobase fragment of the survivin promoter used in this study overlaps with the 5′ portion of the gene studied by Li and Altieri *(6)* and contains the CHR (cell

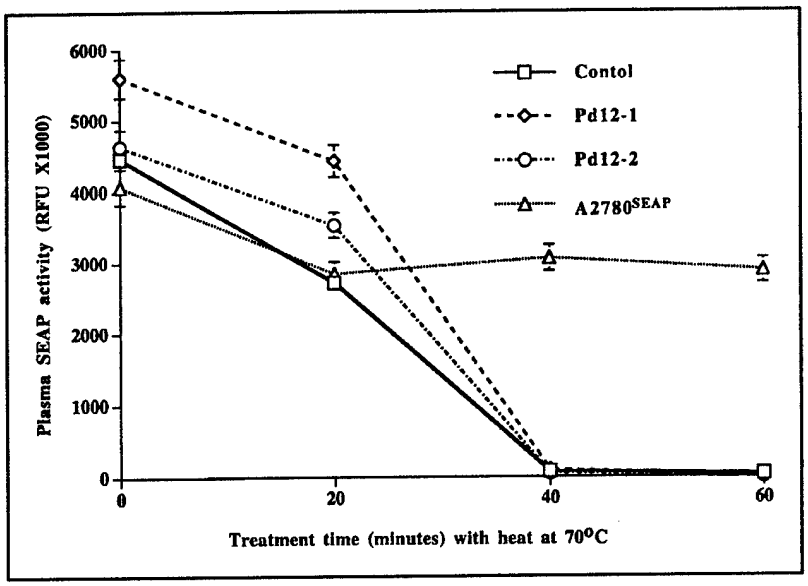

Fig. 5. Heat sensitivity of secreted alkaline phosphatase (SEAP) compared with that of endogenous alkaline phosphatase. Two microliters of plasma was used for each determination except for $A2780^{SEAP}$, where the plasma was diluted 1 : 100. For the control curve, plasma was obtained from a normal C57BL/6 mouse. For the curves Pd12–1 and Pd12–2, plasma was obtained from two C57BL/6 pregnant mice carrying fetuses of age embryonic day 12. For the $A2780^{SEAP}$ curve, plasma was obtained from a CB17/ICR severe combined immunodeficient mouse carrying a $A2780^{SEAP13}$ tumor. Data are the mean of three determinations. **Error bars** are 95% confidence intervals.

cycle gene homology region) and abundant SP_1 and CDE *(6)* as well as E_2F *(12)* transcription factor binding sites that they believe are responsible for controlling the transcription of survivin.

As indicated above, our interest in the survivin promoter first arose because of a desire to drive transgene expression in a cancer-specific manner for cancer gene therapy, to improve gene delivery progress, to specifically regulate expression of transgenes to limit the toxicity of therapeutic genes such as herpes simplex virus thymidine kinase, and to create a tumor-selective replicative oncolytic virus. We believe that the survivin promoter's specificity and expression in many early-stage cancers make it an excellent candidate for these purposes *(17,19)*. Finally, we believe that this cancer-specific reporter gene system could have major implications for monitoring tumor initiation and progression in tumor-prone transgenic animals.

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Notes

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